In vitro production of haploid cells after coculture of CD49f+ with Sertoli cells from testicular sperm extraction in nonobstructive azoospermic patients

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Objective: To isolate CD49f+ cells from testicular sperm extraction (TESE) samples of azoospermic patients and induce meiosis by coculturing these cells with Sertoli cells.
Design: Prospective analysis.
Setting: Research center.
Patient(s): Obstructive azoospermic (OA) and nonobstructive azoospermic (NOA) patients.
Intervention(s): TESE, with enzymatic dissociation of samples to obtain a cell suspension, which was cultured for 4 days with 4 ng/mL GDNF. The CD49f+ cells were sorted using fluorescence-activated cell sorting (FACS) as a marker to identify spermatogonial stem cells (SSCs), which were cocultured with Sertoli cells expressing red fluorescent protein (RFP) in knockout serum replacement (KSR) media with addition of 1,000 IU/mL of follicle-stimulating hormone (FSH), 1 μM testosterone, 40 ng/mL of GDNF, and 2 μM retinoic acid (RA) for 15 days in culture at 37°C and 5% CO_2 to induce meiotic progression. Cells were collected and analyzed by immunofluorescence for meiosis progression with specific markers SCP3 and CREST, and they were confirmed by fluorescence in situ hybridization (FISH).
Main Outcome Measure(s): Isolation of CD49f+ cells and coculture with Sertoli cells, meiosis progression in vitro, assessment of SSCs and meiotic markers real-time polymerase chain reaction (RT-PCR), immunohistochemical analysis, and FISH.
Result(s): The CD49f+ isolated from the of total cell count in the TESE samples of azoospermic patients varied from 5.45% in OA to 2.36% in NOA. Sertoli cells were obtained from the same TESE samples, and established protocols were used to characterize them as positive for SCF, rGDNF, WT1, GATA-4, and vimentin, with the presence of tight junctions and lipid droplets shown by oil red staining. After isolation, the CD49f+ cells were cocultured with RFP Sertoli cells in a 15-day time-course experiment. Positive immunostaining for meiosis markers SCP3 and CREST on days 3 to 5 was noted in the samples obtained from one NOA patient. A FISH analysis for chromosomes 13, 18, 21, X, and Y confirmed the presence of haploid cells on day 5 of the coculture.
Conclusion(s): In vitro coculture of SSCs from TESE samples of NOA patients along with Sertoli cells promoted meiosis induction and resulted in haploid cell generation. These results improve the existing protocols to generate spermatogenesis in vitro and open new avenues for clinical translation in azoospermic patients. (Fertil Steril® 2012;98:580–90. ©2012 by American Society for Reproductive Medicine.)
Key Words: CD49f+ cells, coculture, gametes, germ line, in vitro, meiosis, Sertoli cells, spermatogenesis, spermatogonial stem cell

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Obstructive (OA) and nonobstructive (NOA) azoospermia are major causes of male infertility. Testicular sperm extraction (TESE) is usually performed to search for sperm that will be later used in the intracytoplasmic sperm injection (ICSI) technique (1). Spermatogenesis is normal

in OA patients, so TESE usually solves the problem (2). However, in patients whose NOA is caused by a variety of pathologies that lead to spermatogenesis arrest, their only chance for paternity lies in sperm banks with donor sperm (3).

Essentially, spermatogenesis is a process by which germ cells (GCs) proliferate and differentiate into haploid sperm. Germ cells initially migrate to the basement membrane of the seminiferous tubules of the testis, where they maintain their ability to proliferate and differentiate to sperm under the influence of Sertoli cells throughout a lifetime (4–6).

Accumulated evidence demonstrates the existence of a stem cell population in human adult testes (7–12). Spermatogonial stem cells (SSCs) are the adult stem cell population that renews itself and produces spermatogenic cells after transplantation into the seminiferous tubules of infertile recipient males, constituting a model system to understand spermatogenesis (13). Isolation of the SSC population can be achieved by sorting them by the presence of the cell surface markers CD49f, GPR-125, and GFRA-1 (7, 11, 14, 15) and by further culturing them with the addition of growth factors such as GDNF, LIF, EGF, GFRA-1, or bFGF (11).

In humans, in vitro spermatogenesis from SSCs to obtain haploid sperm has yet to be achieved. Researchers have investigated this process via various strategies such as combinations of growth factors or hormones or/and coculture of germ cells with testicular tissue (16–22). Recently, important advances in the in vivo production of sperm in mice that have resulted in healthy offspring have used two different approaches: the first consists of starting with an epiblast-like cell intermediate that is transplanted into mouse testes (23); the second comprises an organ culture system of SSCs in prepubertal gonadal tissue (24). Our study isolated SSCs from the TESE samples of azoospermic men to induce the meiosis process in vitro by use of a coculture system with Sertoli cells.

MATERIALS AND METHODS

Human Samples

Our study was approved by the institutional review board of the Instituto Valenciano de Infertilidad (IVI) in accordance with Spanish legislation (NCT: 01375662). The samples were obtained after signed, informed consent from the patients. The samples, composed of seminiferous tubules and interstitial tissue, were obtained from 20 azoospermic patients (OA, n = 9; NOA, n = 11) as part of their diagnosis/therapeutic workup. The sperm obtained from the TESE samples was frozen for subsequent use in ICSI, and the residual tissue was transported in basic media and maintained on ice for 60 to 90 minutes after surgery. The tissue was washed three times in Hank's balanced salt solution 1x (HBSS; PAA) with penicillin/streptomycin (Invitrogen); the samples were mechanically dissected out into small pieces (1 mm^3) and enzymatically dissociated with collagenase type IA (1,000 IU/mL; Sigma) for 20 minutes at 37°C on a shaker. Then the samples were incubated with TrypLE Select (Invitrogen) for 10 minutes at 37°C on a shaker, filtered through a 50-μm mesh (Partec), and centrifuged at 1,000 rpm for 5 minutes. The cell pellet was seeded on a plate (maximum of 2×10^6 cell/cm^2 for a culture dish) containing media supplemented with 20% embryonic stem-cell qualified fetal bovine serum (ES-qualified FBS; Invitrogen), plus 4 to 40 ng/mL of GDNF (Sigma); this was incubated at 32°C in 5% CO_2 for 4 days.

CD49f+ Cell Isolation

After 4 days of culture, the media were collected, and the cultured cells were gently washed twice with Dulbecco's modified Eagle medium (DMEM; Invitrogen). They were then washed once with phosphate-buffered saline (PBS; PAA) to recover all the unattached cells and to discard the monolayer adherent cells. The collected cells were centrifuged at 1,000 rpm for 5 minutes, suspended in basic media, and filtered through a 30-μm filter (Partec). The single-cell suspension obtained was then incubated with 5% bovine serum albumin (BSA; Sigma) in PBS for 30 minutes at 4°C, washed with 0.1% BSA in PBS, and incubated with an Alexa Fluor 488 1:100 conjugated antibody against the CD49f surface marker (α6-integrin; Biolegend) on ice for 45 minutes. Peripheral blood lymphocytes were used as positive controls for CD49f, while human skin fibroblast feeder cells were employed as the negative control. The cells that expressed positive for CD49f were separated from the whole population by fluorescence-activated cell-sorting (FACS) (MoFlo Modular Flow Cytometer; Beckman Coulter).

Sertoli Cell Purification

After we had obtained the cell suspension from the TESE samples, the cells were resuspended in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12; GIBCO, Invitrogen) supplemented with 10% FBS; these were seeded into culture plates and incubated at 37°C in 5% CO_2 for 10 minutes. After incubation, the media containing different spermatogenic cell types were removed, and the attached cells were cultured for approximately 1 week; the media were changed every other day until reaching confluence. The cell monolayer was trypsinized with TrypLE Select diluted (1:1) in PBS (PAA) for 5 minutes at 37°C (25). The cell number was calculated, and 1.10^6 cells were then incubated with 0.5 μg/mL Nile red (Sigma) for 15 minutes in PBS. The cells were filtered through a 50-μm sieve (Partec), and cell purification was performed using a cell sorter (MoFlo; Beckman Coulter). The Sertoli cells positive for Nile red staining were seeded at a density of 50,000 cells/well with a collagen matrix (Biocoat Collagen I; Becton Dickinson) in DMEM/F-12 supplemented with 10% FBS, and were cultured for 10 days at 37°C in 5% CO_2. Another demonstration of their nature was performed by oil red staining, electron microscopy for lipid droplet and tight junctions, and polymerase chain reaction for specific markers such as the GDNF receptor (GAF), stem cell factors (SCF), and transcription factors GATA-4 and Wilms tumor (WT-1).

Viral Transduction of Sertoli Cells with Red Fluorescent Protein (RFP)

Sertoli cells were transfected with a lentiviral vector coding for red fluorescent protein (RFP), as previously described by our group (26). Briefly, lentiviral stocks were obtained using the packaging cell line 293-T (American Type Culture Collection,