Eliminating acute lymphoblastic leukemia cells from human testicular cell cultures: a pilot study

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Objective: To study whether acute lymphoblastic leukemia (ALL) cells survive in a human testicular cell culture system.
Design: Experimental laboratory study.
Setting: Reproductive biology laboratory, academic medical center.
Patient(s): Acute lymphoblastic leukemia cells from three patients and testicular cells from three other patients.
Intervention(s): Acute lymphoblastic leukemia cells were cultured alone or in combination with testicular cells, at various concentrations, in a system that has recently been developed to propagate human spermatogonial stem cells.
Main Outcome Measure(s): Viability of ALL and testicular cells during culture was evaluated by flow cytometry using markers for live/dead cells. Furthermore, the presence of ALL cells among testicular cells was determined by highly sensitive (1:10,000 to 1:100,000 cells) patient-specific antigen-receptor minimal residual disease polymerase chain reaction. The presence of spermatogonia at the end of culture was determined by reverse transcription–polymerase chain reaction for ZBTB16, UCHL1, and GPR125.
Result(s): The ALL cells cultured separately did not survive beyond 14 days of culture. When cultured together with testicular cells, even at extremely high initial concentrations (40% ALL cells), ALL cells were undetectable beyond 26 days of culture. Reverse transcription–polymerase chain reaction confirmed the presence of spermatogonia at the end of the culture period.
Conclusion(s): Our pilot study shows that the described testicular cell culture system not only allows for efficient propagation of spermatogonial stem cells but also eliminates contaminating ALL cells. (Fertil Steril® 2014;101: 1072–8. ©2014 by American Society for Reproductive Medicine.)
Key Words: Fertility preservation, childhood cancer, leukemia, spermatogonial stem cell, human testis

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The survival rate of children with cancer has continuously improved over the past few decades, and the 5-year event-free survival rates are currently approximately 80% (1–3). Gonadal damage is a relatively common consequence of the treatments used to cure pediatric cancer, and many cancer survivors are sterile (4, 5). Currently, in prepubertal boys with cancer, testicular biopsies taken before the onset of cancer

treatment are being cryopreserved in several centers to preserve fertility (6–9).

Future use of this tissue will involve thawing of the biopsy, followed by in vitro propagation of spermatogonial stem cells (SSCs) and autotransplantation of these cultured cells when infertility has manifested itself (10). We recently described a testicular cell culture system that enables propagation of adult and prepubertal human SSCs necessary for efficient transplantation (11, 12).

One of the most important safety issues that still needs to be addressed is the elimination of remaining malignant cells in testicular biopsies in the case of nonsolid tumors. The most common nonsolid cancer in prepubertal children is acute lymphoblastic leukemia (ALL), constituting up to 40% of the main tumors in children under the age of 14 years (13). In a single report infiltration of leukemic cells in the testis as proven by histologic examination was described in up to 30% of boys with ALL (14). Although precise data are not available, the percentage of leukemic cells in the testis of boys with ALL is unlikely to exceed 1% of all cells (15). A few studies have attempted to eliminate malignant cells from mouse, nonhuman primate, and human testicular cell suspensions (16–20). These studies are all based on cell sorting techniques and show contradicting results.

To investigate the persistence of contaminating malignant cells in the previously described culture system (11, 12), we cultured ALL cells separately and in combination with testicular cells in various concentrations and used highly sensitive methods to detect the presence of ALL cells at various time points during culture.

MATERIALS AND METHODS

Cell Preparation and Culture

Malignant cells were obtained by bone marrow aspirations from three patients (SQ8512, SQ9610, and SQ11485) with B-cell ALL during their diagnostic process. The percentages of ALL cells before and after cryopreservation in these cell suspensions were evaluated according to their specific phenotypes and percentage of epitope expression, as determined by flow cytometry with a panel of multiple antibodies (CD1, -2, -3, -5, -10, -19, -20, and -34) (21, 22). This percentage, which varied between 77% and 94%, was used to correct for the number of ALL cells to reach the desired percentage of living ALLs cells at the start point of any culture (i.e., 0, 0.04%, 0.4%, 4%, and 40%). The numbers of ALL and testicular cells were also corrected for viability using trypan blue staining at the start of every culture, so that the ratios described always reflected the percentage of vital and alive cells.

Testicular cells were isolated from the donated testis tissue from one adult man who underwent castration as part of the prostate cancer treatment (URO0126) and from two prepubertal boys with Hodgkin lymphoma who stored their testis biopsy before chemotherapy (URO0113 and URO0114, 8- and 6.5-year-old boys, respectively) (12). Samples were used for research after receiving oral informed consent from the adult man and written informed consent from the parents of the prepubertal boys (12). According to Dutch law, ethics committee approval was not required, because anonymized tissue samples (URO0126, SQ8512, SQ9610, and SQ11485) were used. The local institutional review board (Avicenna Research Institute) approved collection and use of URO0113 and URO0114 samples in this research. Pathologic evaluations of the testicular biopsy from the included boys with Hodgkin lymphoma showed no evidence of malignant cells. To increase the number of testicular cells, these cells were cultured for 3–5 weeks before initiation of the experiments in our recently established testicular cell culture condition based on supplemented StemPro medium (#10639-011, Invitrogen) in noncoated dishes as previously described (11, 12). Testicular cells were then cocultured with ALL cells at various concentrations (percentage viable ALL/testicular cells: 0, 0.04%, 0.4%, 4%, and 40%) in the same condition as culturing the testicular cells alone. The medium was refreshed every 3 to 4 days with centrifugation to also collect floating cells. When cells became 90%–100% confluent, cells were passaged (average 1:6) using trypsin ethylenediaminetetraacetic acid (0.25%) (Invitrogen) and transferred to new dishes in a concentration of 10,000–15,000 cells per cm^2. During each passage, surplus cells were used for DNA and RNA isolation. Because at least 35 days of culture are required to propagate enough SSCs for autotransplantation, we cultured the ALL/testicular cell mixes of all three patients for 24–52 days.

Viability of ALL and Testicular Cells during Culture

To examine viability of ALL cells from three patients (SQ8512, SQ9850, and SQ11485) during culture for a period of 14–16 days, an equal amount of cells was plated in each well of a 48-well plate (30,000 cells per well). During this 14–16-day culture period, every 24 hours cells from three wells were harvested by cell scraping (#3010, Corning) and evaluated by flow cytometry with a live–dead kit based on Calcein AM and Ethidium H1 staining according to manufacturer's instructions (MP 3224, Invitrogen). As a control, parallel cultured human testicular cells (URO0126) were used.

Minimal Residual Disease Polymerase Chain Reaction to Trace ALL Cells

To detect the leukemic cells in the cocultures with testicular cells, a leukemia-specific polymerase chain reaction (PCR) was developed for each patient on the basis of patient-specific antigen receptor rearrangements present in the leukemic cells, as previously described (23–25). Moreover, to minimize the theoretical risk of false negativity by the outgrowth of subclones of leukemic cells during the culture period, two patient-specific antigen receptor targets were selected for each patient.

Deoxyribonucleic acid (DNA) from ALL cells mixed with testicular cells before and during culture was extracted using the QIAamp DNA Mini Kit (51306, Qiagen) (26) with an elution volume of 55 μL instead of 200 μL to increase the concentration of DNA. Extracted DNA was measured by the fluorometer Qubit (Invitrogen) using the DNA HS assay kit (Q32854, Invitrogen). Polymerase chain reaction was performed as previously described (27), using the ABI PRISM

7700 Sequence Detection System containing a 96-well thermal cycler (PE Biosystems). Reaction mixtures of 50 μL contained TaqMan buffer A, 5 mM $MgCl_2$, 200 mM each 2′-deoxynucleoside 5′-triphosphates (dNTPs), 900 nM primers, 100 nM probe, 1.25 U AmpliTaq Gold (PE Biosystems), 10% glycerol, and 500 ng DNA. The reaction conditions were 10 minutes at 95°C followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C–69°C. All quantitative PCR experiments were performed in triplicate.

To verify that in a mixture of ALL cells and testicular cells the same quantitative range and sensitivity of the minimal residual disease (MRD)-PCR is reached as when ALL cells are mixed with mononuclear blood cells, 10-fold dilution series of ALL cells in testicular cells and in mononuclear blood cells were compared. Sensitivity and quantitative range of the respective PCR assays was determined according to the guidelines of the European Study Group on MRD detection in ALL (28). The sensitivity of the PCR ranged from 10^{-4} (SQ9610 and SQ11485) to 10^{-5} (SQ8512), and the amount of leukemic cells could be reliably quantified up to 10^{-4} (SQ8512, SQ9610, and SQ11485) (29, 30).

Gene Expression

To determine the presence of spermatogonia cells, total RNA from cultured mixed cells and whole testis (positive control) was isolated using the RNeasy Mini Kit (74104, Qiagen). Extracted RNA was measured by Qubit analysis (Invitrogen) using the RNA assay kit (Q32855, Invitrogen). For reverse transcription–PCR, first-strand complementary DNA was synthesized with random hexamers and the Superscript II preamplification system (Invitrogen). Polymerase chain reaction was carried out with specific primers for spermatogonial markers (31, 32) *ZBTB16* (*PLZF*) (forward: GGTCGAGCTTCCTGATAACG; reverse: CCTGTATGTGAG CGCAGGT; product size: 396 bp), *UCHL1* (*PGP9.5*) (forward: ATGCCGTGGCACAGGAAGGC; reverse: GGCAGCGTCC TTCAGCAGGG; product size: 164 bp), and *GPR125* (forward: GTGGCTGGCGCAGAGGGAAG; reverse: CCACCGGGCTG CAACAGCAT; product size: 610 bp) and housekeeping gene *TBP* (Tata box binding protein) (forward: GTGACCC AGCAGCATCACTG; reverse: GTCATGGCACCCTGAGGG; product size: 224 bp) as a general marker. Polymerase chain reaction amplification was performed on cDNA (with reverse transcriptase) and on RNA (without reverse transcriptase) as follows: 3 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at specific annealing temperature for each primer set (*ZBTB16*, 55°C; *UCHL1*, 65°C; *GPR125*, 65°C; and *TBP*, 59°C), 1 minute at 72°C, and a final elongation of 5 minutes at 72°C.

RESULTS

When cultured separately in testicular cell culture conditions (Fig. 1A and B), ALL and testicular cells showed dramatic differences in cell survival. The percentage of vital ALL cells of all three patients showed a dramatic decrease during the first 3–6 days of culture. After 14 days of culture all ALL cells were dead as measured by flow cytometry analyses for vital and dead cells, whereas testicular cells cultured in parallel proliferated well even after 8 weeks of culture (Fig. 2).

Survival of ALL cells in the presence of testicular cells (Fig. 1C) was subsequently investigated using real-time quantitative PCR analysis of the "leukemia-specific" junctional regions of rearranged antigen receptor genes known as MRD-PCR. In each patient we were able to detect a single ALL cell in the presence of 10^4 or 10^5 testicular cells, and the percentage of ALL cells could be accurately quantified up to a concentration of 10^{-4} in all three patients (Supplemental Fig. 1, available online).

At the time point of the first passage, after 10–16 days of culture, ALL cells were undetectable in the cultures with an initial concentration on 0.04%, 0.4%, and 4% in two out of three patients. At the highest initial concentration (40%) ALL cells could be detected in all three patients at this time point, but the level of contamination with ALL cells was extremely low and was within the quantitative range of the assay in only a single patient. At the second passage, after 20–26 days, ALL cells were undetectable in all initial concentrations of all patients (Table 1). On the basis of these PCR results, a 1.5 log reduction was obtained during the first 10–16 days of culture and at least a 2 log reduction during the first 20–26 days.

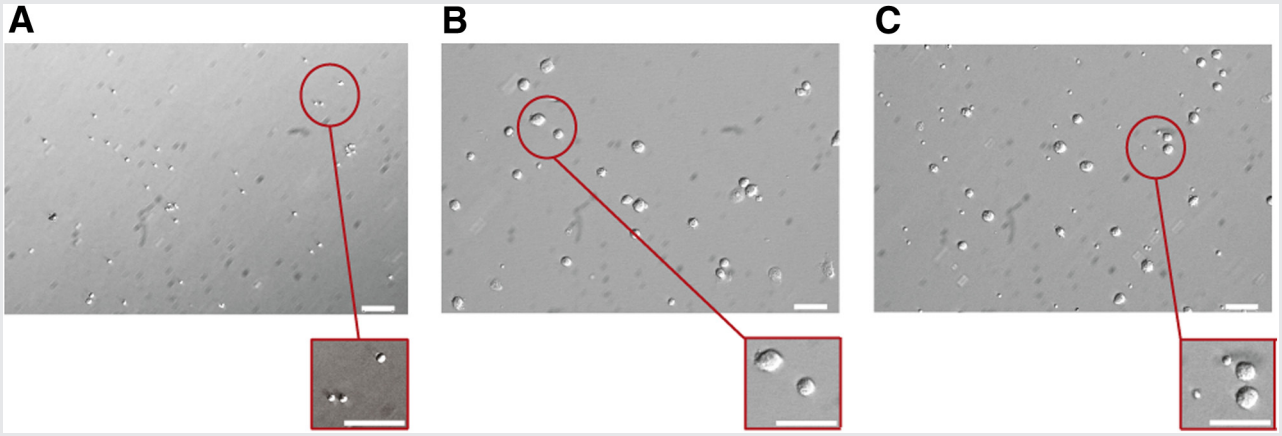
Reverse transcription–PCR using *ZBTB16*, *UCHL1*, and *GPR125* (markers specific for spermatogonia) confirmed the presence of spermatogonia at the end of the culture period (Fig. 3).

DISCUSSION

This study demonstrates that our recently established SSC culture system for propagating human SSCs efficiently eliminates contaminating malignant ALL cells.

We found no vital ALL cells beyond 14 days of culture when ALL cells were cultured separately under human testicular culture conditions, as demonstrated by flow cytometric analyses for vital and dead cells. In the mixed ALL/testicular cell cultures, no ALL cells could be detected from the second passage onward at 20–26 days in culture, as measured by MRD-PCR, even when the initial percentage of ALL cells far exceeded (40%) the percentage of ALL cells one would expect to find in testicular tissue of a boy with ALL. Because cells were diluted at each passage with a ratio of 1:6, the question then arises whether this elimination is based on simple dilution or by a real reduction in numbers. If simple dilution would have occurred, the MRD-PCR used in the present study would have been sensitive enough to detect the diluted leukemic cells until after the 4th (day 41–47), 3rd (day 31–37), or 2nd passage (day 20–26) in cultures initiated with 40%, 4%, or 0.4% ALL cells, respectively. Because no ALL cells were detected at the 2nd passage with cultures initiated with 40% ALL cells, the percentage of ALL cells during culture is not reduced as a result of dilution but truly by a reduction in the number of ALL cells during culture. Although this study included only a limited number of patients and could thus be considered as a pilot study, the consistency of the results between the patients in our view clearly shows that ALL cells do not survive in the described testicular culture condition.

FIGURE 1



Acute lymphoblastic leukemia cells and testicular cells in culture (SQ8512/URO113). ALL cells (A) and testicular cells (B) were seeded separately and together (C) (percentage viable ALL/testicular cells = 40%) for culturing in human testicular cell culture condition in medium used to propagate human SSCs. Scale bars = 50 μm.

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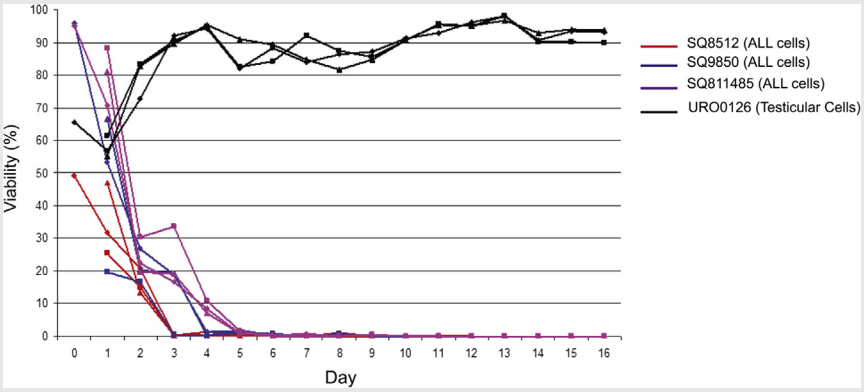
Several previous studies, all based on cell sorting methods, assessed the ability to remove leukemic or lymphoma cells from uncultured human spermatogonia/testicular cells. The first study, in which eight leukemia and lymphoma cell lines were used, showed that negative selection for both major histocompatibility complex (MHC) class I and CD45 markers using fluorescence-activated cell sorting (FACS) was effective for all cell lines except the erytroleukemic K562 cell line, whereas successful elimination of this cell line required incubation with interferon-γ before FACS sorting (16). The second study showed ineffective elimination of human malignant cells by negative selection only for MHC class I by FACS (17). The third study, by isolating EpCAM(+)/HLA-ABC(−)/CD49e(−) cells via FACS sorting, showed the enrichment of human spermatogonia and removal of malignant contamination. The purity of this FACS method to remove malignant cells was tested by the human to nude mouse tumor assay, which has a sensitivity to detect a 0.2% contamination with cancer cells (10 cells in a transplanted fraction of 5,000 cells) (20). However, the gold standard to detect remaining malignant cells in the context of bone marrow transplantation is MRD-PCR (19). Indeed, the MRD-PCR used in our present study was able to detect the presence of ALL cells among cultured testicular cells with very high sensitivity (0.01%–0.001%).

In the fourth study, the combination of positive selection for CD49f and negative selection for MHC class I markers and subsequently cell selection by matrix adhesion molecules (collagen I and laminin) was insufficient to eliminate all malignant cells (18). This study assessed the ability to remove ALL cells from a mixture of ALL and human testicular cells after culture for a period of 4 days before magnetic-activated cell

FIGURE 2



Survival of ALL cells in vitro. Viability of ALL cells and testicular cells in human testicular cell culture conditions during 16 days of culture. Data are from flow cytometric analysis using a dead–alive assay kit. Every day, three measurements (from three wells) for each cell line are shown.

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TABLE 1

Overview of the MRD-PCR results in different passages of cell culture.

Percentage of ALL cells in coculture with testicular cells	ALL patient SQ11485 Testicular cells patient URO0126	ALL patient SQ8512 Testicular cells patient URO0113	ALL patient SQ9610 Testicular cells patient URO0114
0	— (0) — (10) — (20) — (31) — (41) — (52)	— (0) — (16) — (26) — (37) — (47)	— (0) — (10) — (24)
0.04	PNQ (0) — (10) — (20) — (31) — (41) — (52)	0.08%[a] (0) — (16) — (26) — (37) — (47)	0.03%[a] (0) PNQ (10) — (24)
0.4	0.1%[a] (0) — (10) — (20) — (31) — (41) — (52)	0.3%[a] (0) — (16) — (26) — (37) — (47)	0.76%[a] (0) PNQ (10) — (24)
4	1.7%[a] (0) — (10) — (20) — (31) — (41) — (52)	2.9%[a] (0) — (16) — (26) — (37) — (47)	5.64%[a] (0) PNQ (10) — (24)
40	10%[a] (0) PNQ (10) — (20) — (31) — (41) — (52)	19%[a] (0) PNQ (16) — (26) — (37) — (47)	35.8%[a] (0) 0.4%[a] (10) — (24)

Note: For each patient, two patient-specific antigen receptor targets were selected for MRD-PCR, and results of expression of both targets were in all cases comparable to each other, with a correlation coefficient of 0.9, but always the highest level of expression was mentioned in this table. Symbol "—" represents absence of any specific MRD-PCR signal. Day of culture is shown in parentheses. PNQ = positive not quantifiable (i.e., a positive signal outside the quantitative range).

[a] The percentage of detected ALL cells based on MRD-PCR signal.

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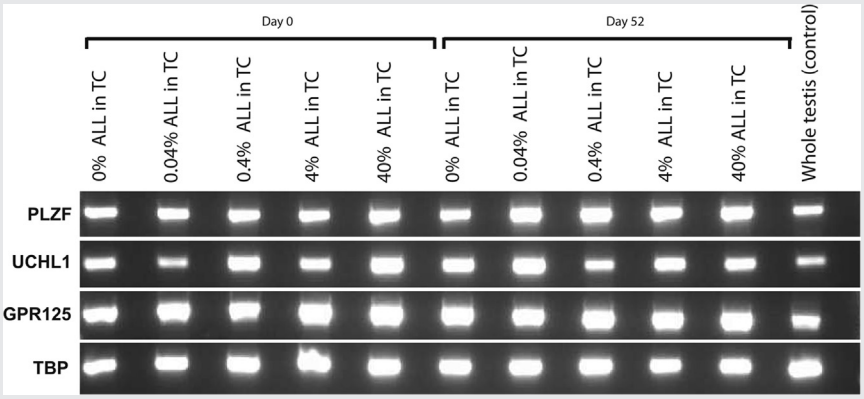
sorting and demonstrated that this period of culture was inefficient in eliminating the leukemic cells (18). This is in line with our study: we demonstrate that ALL cells mixed with testicular cells from three ALL patients are still detectable after a few days of culture but are dead after 14 days of culture and completely eliminated after 20–26 days of culture.

How many malignant cells are necessary to reintroduce cancer upon SSC autotransplantation? The minimal number of malignant cells capable of reintroducing cancer in the context of testicular autotransplantation was investigated in only one study. This study demonstrated that intraluminal injection of as few as 20 rat leukemia cells into the testes of recipient rats could induce disease relapse in three of five animals (33). In all three patients in the present study, we observed a 1.5–2 log reduction at the first and second passages of culture, whereas SSCs have to be cultured for approximately 35–50 days (approximately three to five passages) to obtain enough cells for efficient autotransplantation from a biopsy of 0.2 mL (11, 12). If the log reduction is on the same order of magnitude during subsequent passages, the risk of transplanting leukemic cells is highly unlikely.

Obviously no test can guarantee that all ALL cells are fully eliminated, but the MRD-PCR assays we have applied in this study are at present the most sensitive assays for this purpose. Because a PCR reaction cannot discriminate between viable and dead cells, the absence of any positive signal makes the presence of viable leukemic cells capable of reintroducing cancer even more unlikely. The facts that ALL cells were incapable of surviving for more than 14 days of culture and no (dead or vital) ALL cells could be detected beyond 20–26 days of coculture with testicular cells even if the initial concentration of ALL cells was 40% clearly indicate that no vital ALL cells will be present if SSCs are propagated in our culture system for the required 35–50 days between passages 3 and 5, respectively (12).

In our experiments we used ALL cells derived from the bone marrow of ALL patients. Theoretically, ALL cells that are present in the testis might behave differently, although there are no real data to support this. In addition, it is impossible to derive testicular ALL cells in sufficient number to perform the experiments described here. We therefore feel it is justified to say that our setup was as close as possible to

FIGURE 3



Expression of spermatogonial markers overtime in testicular cultured cells (TC) (URO 0126) with various percentages of ALL (SQ11485) at the start (day 0) and at the end of culture (day 52). Reverse transcription–PCR of whole testis from human testis from the same study participant (URO126) was used as a positive control for spermatogonial markers. TBP (Tata box binding protein) was used as a loading reference marker. The second lane for each sample tested shows the negative (without reverse transcription) control for genomic contamination.

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the future clinical scenario of performing SSC autotransplantation in a survivor of childhood ALL.

It is known that various leukemia cell lines can behave differently in vitro. Although in this study we have tested leukemia cells from three different patients, it might be that other cells behave differently, and therefore it will be beneficial to test more cell lines from different types of leukemia in the future. Because the results could theoretically vary between patients and between cultures, we do suggest that when SSC autotransplantation will be applied clinically in the future, testing for the presence of ALL cells before transplantation should be an integral part of the procedure. The described MRD-PCR method has proven to be useful for this purpose. A theoretical alternative way to address the risk of reintroducing leukemia cells with SSC transplantation could be ex vivo spermatogenesis using the cryopreserved testicular biopsy followed by intracytoplasmic sperm injection of the generated spermatozoa. Although this method is not yet feasible for human testis biopsies, the method is successfully used for neonatal mouse testes (34). This alternative method does require the hormonal treatment of the female partner to retrieve mature oocytes for intracytoplasmic sperm injection, whereas SSC autotransplantation theoretically will enable in vivo conception and life-long spermatogenesis and fertility.

Taken together, ALL cells do not survive in our recently established human testicular culture system for propagation of SSCs. These results are a step toward clinical application of autotransplantation of in vitro–propagated SSCs derived from a small testis biopsy of prepubertal boys with nonsolid cancers. In light of our findings, all boys with cancer, including those with nonsolid tumors, should be offered the possibility of storing testicular tissue for possible future clinical use.

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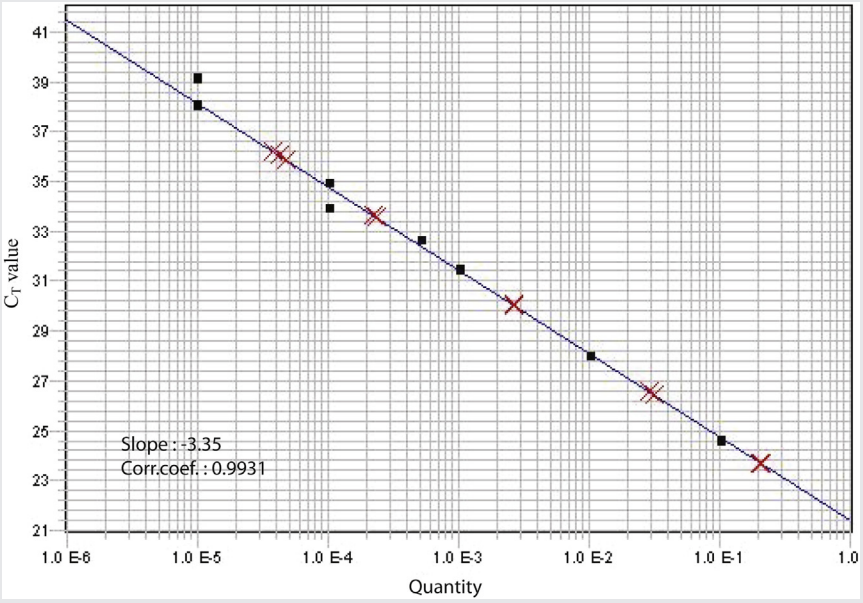
REFERENCES

1. Mertens AC, Yasui Y, Neglia JP, Potter JD, Nesbit ME Jr, Ruccione K, et al. Late mortality experience in five-year survivors of childhood and adolescent cancer: the Childhood Cancer Survivor Study. J Clin Oncol 2001;19:3163–72.
2. Vadaparampil S, Quinn G, King L, Wilson C, Nieder M. Barriers to fertility preservation among pediatric oncologists. Patient Educ Couns 2008;72:402–10.
3. Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. J Clin Oncol 2011;29:551–65.
4. Rivkees SA, Crawford JD. The relationship of gonadal activity and chemotherapy-induced gonadal damage. JAMA 1988;259:2123–5.
5. Meistrich ML, Wilson G, Brown BW, da Cunha MF, Lipshultz LI. Impact of cyclophosphamide on long-term reduction in sperm count in men treated with combination chemotherapy for Ewing and soft tissue sarcomas. Cancer 1992;70:2703–12.
6. Kvist K, Thorup J, Byskov AG, Hoyer PE, Mollgard K, Yding Andersen C. Cryopreservation of intact testicular tissue from boys with cryptorchidism. Hum Reprod 2006;21:484–91.
7. Keros V, Hultenby K, Borgstrom B, Fridstrom M, Jahnukainen K, Hovatta O. Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod 2007;22:1384–95.

8. Ginsberg JP, Carlson CA, Lin K, Hobbie WL, Wigo E, Wu X, et al. An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. Hum Reprod 2009; 25:37–41.
9. Wyns C, Curaba M, Petit S, Vanabelle B, Laurent P, Wese JF, et al. Management of fertility preservation in prepubertal patients: 5 years' experience at the Catholic University of Louvain. Hum Reprod 2011;26:737–47.
10. Brinster RL. Male germline stem cells: from mice to men. Science 2007;316: 404–5.
11. Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, et al. Propagation of human spermatogonial stem cells in vitro. JAMA 2009;302:2127–34.
12. Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt AM. In vitro propagation of human prepubertal spermatogonial stem cells. JAMA 2011;305:2416–8.
13. Steliarova-Foucher E, Stiller C, Kaatsch P, Berrino F, Coebergh JW, Lacour B, et al. Geographical patterns and time trends of cancer incidence and survival among children and adolescents in Europe since the 1970s (the ACCISproject): an epidemiological study. Lancet 2004;364:2097–105.
14. Akhtar M, Ali MA, Burgess A, Aur RJ. Fine-needle aspiration biopsy (FNAB) diagnosis of testicular involvement in acute lymphoblastic leukemia in children. Diagn Cytopathol 1991;7:504–7.
15. Mandrell BN, Pritchard M. Understanding the clinical implications of minimal residual disease in childhood leukemia. J Pediatr Oncol Nurs 2006;23: 38–44.
16. Fujita K, Tsujimura A, Miyagawa Y, Kiuchi H, Matsuoka Y, Takao T, et al. Isolation of germ cells from leukemia and lymphoma cells in a human in vitro model: potential clinical application for restoring human fertility after anticancer therapy. Cancer Res 2006;66:11166–71.
17. Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H. The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. Hum Reprod 2007;22:733–42.
18. Geens M, Goossens E, Tournaye H. Cell selection by selective matrix adhesion is not sufficiently efficient for complete malignant cell depletion from contaminated human testicular cell suspensions. Fertil Steril 2011; 95:787–91.
19. Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE. Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. Hum Reprod 2011;26:3222–31.
20. Dovey SL, Valli H, Hermann BP, Sukhwani M, Donohue J, Castro CA, et al. Eliminating malignant contamination from therapeutic human spermatogonial stem cells. J Clin Invest 2013;123:1833–43.
21. Stewart CC, Behm FG, Carey JL, Cornbleet J, Duque RE, Hudnall SD, et al. U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: selection of antibody combinations. Cytometry 1997;30:231–5.
22. Borowitz MJ, Bray R, Gascoyne R, Melnick S, Parker JW, Picker L, et al. U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: data analysis and interpretation. Cytometry 1997;30:236–44.
23. Bruggemann M, van der Velden VH, Raff T, Droese J, Ritgen M, Pott C, et al. Rearranged T-cell receptor beta genes represent powerful targets for quantification of minimal residual disease in childhood and adult T-cell acute lymphoblastic leukemia. Leukemia 2004;18:709–19.
24. van der Velden VH, Wijkhuijs JM, Jacobs DC, van Wering ER, van Dongen JJ. T cell receptor gamma gene rearrangements as targets for detection of minimal residual disease in acute lymphoblastic leukemia by real-time quantitative PCR analysis. Leukemia 2002;16:1372–80.
25. van der Velden VH, Willemse MJ, van der Schoot CE, Hahlen K, van Wering ER, van Dongen JJ. Immunoglobulin kappa deleting element rearrangements in precursor-B acute lymphoblastic leukemia are stable targets for detection of minimal residual disease by real-time quantitative PCR. Leukemia 2002;16:928–36.
26. Verhagen OJ, Wijkhuijs AJ, van der Sluijs-Gelling AJ, Szczepanski T, van der Linden-Schrever BE, Pongers-Willemse MJ, et al. Suitable DNA isolation method for the detection of minimal residual disease by PCR techniques. Leukemia 1999;13:1298–9.
27. Verhagen OJ, Willemse MJ, Breunis WB, Wijkhuijs AJ, Jacobs DC, Joosten SA, et al. Application of germline IGH probes in real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia. Leukemia 2000;14:1426–35.
28. van der Velden VH, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia 2007;21:604–11.
29. van Dongen JJ, Seriu T, Panzer-Grumayer ER, Biondi A, Pongers-Willemse MJ, Corral L, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet 1998;352:1731–8.
30. Steenbergen EJ, Verhagen OJ, van Leeuwen EF, van den Berg H, Behrendt H, Slater RM, et al. Prolonged persistence of PCR-detectable minimal residual disease after diagnosis or first relapse predicts poor outcome in childhood B-precursor acute lymphoblastic leukemia. Leukemia 1995;9:1726–34.
31. Aponte PM, van Bragt MP, de Rooij DG, van Pelt AM. Spermatogonial stem cells: characteristics and experimental possibilities. Apmis 2005;113:727–42.
32. Ebata KT, Yeh JR, Zhang X, Nagano MC. The application of biomarkers of spermatogonial stem cells for restoring male fertility. Dis Markers 2008; 24:267–76.
33. Jahnukainen K, Hou M, Petersen C, Setchell B, Soder O. Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. Cancer Res 2001;61:706–10.
34. Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, et al. In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun 2011;2:472.

SUPPLEMENTAL FIGURE 1



Standard curve constructed from the quantitative PCR (MRD-PCR). The MRD-PCR was able to detect a single ALL cell in the presence of 10^5 testicular cells, and the level of contamination could be quantified up to a concentration of 10^{-4}. *Red crosses* and *black boxes* represent cultured cells and standards, respectively.

Sadri-Ardekani. Eliminating ALL cells in SSC culture. Fertil Steril 2014.