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

Hooman Sadri-Ardekani, M.D., Ph.D., a,b Christa H. Homburg, M.Sc., c Toni M. M. van Capel, B.Sc., d Henk van den Berg, M.D., Ph.D., e Fulco van der Veen, M.D., Ph.D., a C. Ellen van der Schoot, M.D., Ph.D., c Ans M. van Pelt, Ph.D., a and Sjoerd Repping, Ph.D. a

a Center for Reproductive Medicine, Women’s and Children’s Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; b Reproductive Biotechnology Research Center, Avicenna Research Institute, The Academic Center for Education, Culture and Research (ACECR), Tehran, Iran; c Experimental Immunohematology, Sanquin Research at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands; d Departments of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; and e Department of Pediatric Oncology, Women’s and Children’s Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

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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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 ALL 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 (UR00126) 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 (UR00126, SQ8512, SQ9610, and SQ11485) were used. The local institutional review board (Avicenna Research Institute) approved collection and use of UR00113 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 cm². 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 (UR00126) 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
dead as measured by all three patients showed a dramatic decrease during the differences in cell survival. The percentage of vital ALL cells of CGCAGGT; product size: 396 bp), GGTCGAGCTTCCTGATAACG; reverse: CCTGTATGTGAG synthesized with random hexamers and the Superscript 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: GTGTCGACCTCTGATAACG; reverse: CCTGTATGTGAG CGCAGGT; product size: 396 bp), UCHL1 (PGP9.5) (forward: ATGCCGTGGCACAGGAAGGC; reverse: GGCAGCGTCC TTCAGCAGGG; product size: 164 bp), and GPR125 (forward: GTGCTCTGCGGAGAGGAAG; reverse: CACCGGGCTG CAACAGCAT; product size: 610 bp) and housekeeping gene TBP (Tata box binding protein) (forward: GTGACCC AGCAGCATCAGT; reverse: GTCAATGCCAACCTGAGGG; 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.
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 sorting.

![FIGURE 1](image1.png)

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.


![FIGURE 2](image2.png)

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.

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.

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

### TABLE 1

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

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<th>Percentage of ALL cells in coculture with testicular cells</th>
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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.

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 tests (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


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.