ORIGINAL ARTICLE: FERTILITY PRESERVATION

Evaluation of a human ovarian follicle isolation technique to obtain disease-free follicle suspensions before safely grafting to cancer patients

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Objective: To evaluate the safety of our follicle isolation procedure in a model of ovarian tissue artificially contaminated with cancer cells, then to improve the procedure to effectively eliminate malignant cells from follicle suspensions without altering viability.
Design: Prospective experimental study.
Setting: Gynecology research unit in a university hospital.
Patient(s): Ten women undergoing laparoscopy for benign gynecologic disease.
Intervention(s): Follicle isolation from ovarian tissue artificially contaminated with marked fluorescent leukemic cells, either by the usual pickup technique without further treatment (group 1) or by washing three times after pickup (group 2).
Main Outcome Measure(s): Evidence of leukemic cells in follicle suspensions using fluorescence microscopy and quantitative real-time polymerase chain reaction, and analysis of follicle viability.
Result(s): In group 1, 196 leukemic cells were detected by fluorescence microscopy out of 499 follicles retrieved, while just one leukemic cell was found among 772 follicles after three washes. The BCR-ABL fusion transcript was detected when at least 19 cells were present in follicle suspensions; four samples were positive in group 1, and all were negative in group 2. Follicle viability was similar in both groups (95.6% vs. 96.4%).
Conclusion(s): Cancer cells could inadvertently be picked up with isolated follicles in case of malignant contamination of ovarian tissue. A simple purging procedure consisting of three washes proved effective for eliminating leukemic cells while maintaining good follicle viability. (Fertil Steril® 2015;■:■–■. ©2015 by American Society for Reproductive Medicine.)
Key Words: Follicle isolation, leukemia, malignant cell purging, minimal disseminated disease, ovarian follicles, ovarian tissue cryopreservation

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Advances in cancer treatments and aggressive chemotherapy and radiotherapy have led to an increase in survival and life expectancy of young cancer patients. Indeed, the 5-year relative survival rate in children, adolescents, and young adults increased from approximately 60% in 1975 to 82%–86% in 2009 (1). As of January 1, 2010, there were approximately 380,000 survivors of childhood

and adolescent cancer (diagnosed at age 0 to 19 years) still alive in the United States (2).Unfortunately, in many women these life-saving treatments lead to early menopause and subsequent infertility, depending on the age of the patient, her follicular reserve, and the type and dose of drugs used (3–6). Embryo or oocyte cryopreservation may be performed when chemotherapy can be delayed by a minimum of 2–3 weeks, the time required for ovarian stimulation (7, 8). These options are not, however, applicable to prepubertal girls or patients requiring immediate chemotherapy (8–11). For these young women, ovarian tissue cryopreservation followed by orthotopic transplantation has emerged as a promising approach to safeguard fertility, resulting in restoration of endocrine function in most patients and more than 40 live births to date (10,12–15).

Leukemia is the most frequently encountered malignancy in children (0–14 years) representing 26% of all tumors diagnosed at this age (16). According to Wallace et al. (17), the risk of premature ovarian failure after cancer treatment in these young patients is low (<20%) in case of acute lymphoblastic leukemia and moderate (20%–80%) in case of acute myeloblastic leukemia. A non-negligible percentage of these patients will require bone marrow transplantation or further gonadotoxic treatment for incomplete remission or disease recurrence, and will thus change categories to high risk (>80% risk of premature ovarian failure) (18). It is therefore important to offer fertility preservation options to these patients before cancer treatment. The only option currently available for these often prepubertal patients requiring immediate chemotherapy is ovarian tissue cryopreservation.

Ovarian tissue cryopreservation is usually performed before cancer treatment to avoid the harmful effects of chemotherapy and/or radiotherapy on primordial follicles. Hematologic diseases along with breast cancer represent the most frequent indications for ovarian tissue cryopreservation in most centers (19–22). However, the safety of ovarian tissue autotransplantation in patients with leukemia is a major concern. Indeed, experimental studies by four different groups have demonstrated, by sensitive polymerase chain reaction (PCR) or flow cytometry analysis, that cryopreserved ovarian tissue from patients with leukemia may harbor leukemic cells in >50% of cases and (23–26) subsequently transmit the disease, at least in a xenografting model (24).

For these patients at risk of minimal disseminated disease, follicle culture with in vitro maturation may be a solution (27–32). However, despite advances in this field, viable embryos and live offspring have only been obtained in mice with this technique (33–35), and major hurdles remain before improved culture systems in humans yield competent human oocytes (29). Another approach to restore fertility in these patients is grafting of isolated follicles enzymatically purified from frozen-thawed ovarian tissue. Indeed, ovarian follicles are enclosed in a basement membrane that prevents direct contact between follicular cells on the one hand, and capillaries, white blood cells, and nerve processes on the other (36).

With the objective of grafting isolated preantral follicles (obtained from frozen-thawed ovarian tissue) for the purposes of fertility restoration, procedures were set up by our team to isolate (37), recover (38), and transplant (39, 40) human ovarian follicles. A technique for isolation of human preantral follicles following good manufacturing practice conditions was recently optimized by our team with a view toward clinical application (41).

However, even if isolation of preantral follicles from ovarian cortical fragments of healthy women is now a well-established protocol in our department, for patients with leukemia who are at risk of ovarian involvement, malignant cell contamination of the isolated follicle suspension cannot be excluded. Before these follicles can be grafted to patients with leukemia, it is crucial to make sure that the follicle suspension is free of malignant cells.

As cryopreserved ovarian tissue from leukemia patients is not abundantly available and is therefore precious material, we designed a model of ovarian tissue suspensions from healthy patients artificially contaminated with leukemic cells for this preliminary study. Our study evaluated the safety of our follicle pickup technique and set up a protocol to obtain leukemia cell-free follicle suspensions while maintaining good follicle viability. The sensitivity of the PCR technique and its applicability to follicle suspensions was also investigated in this study.

MATERIALS AND METHODS

Use of human ovarian tissue for this study was approved by the institutional review board of the Université Catholique de Louvain (IRB, 2012, 125). Ovarian biopsies were taken from 10 women (between 22 and 36 years of age) undergoing laparoscopic surgery for benign gynecologic disease, after obtaining their informed consent.

Supplemental Figure 1 (available online) shows the experimental design of the study. Ten fresh human ovarian biopsy samples were enzymatically dissociated. A leukemic cell line (BV-173) was marked with a fluorescent cell tracer (CFDA-SE) and added to the resulting ovarian cell suspension. Follicles were retrieved and divided into two groups: in group 1, follicles were picked up by our usual technique without further treatment; in group 2, follicles were washed three times after pickup. Follicle suspensions in both groups were then investigated for the presence of leukemic cells using fluorescence microscopy as well as PCR. Follicle viability was also analyzed in both groups.

Preparation of Leukemic Cells

The BV-173 cell line was derived from a patient with Philadelphia chromosome-positive acute lymphoblastic leukemia (BCR-ABL b2-a2 fusion gene). This cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ no. ACC 20) and cultured in RPMI 1640 medium + 10% fetal bovine serum (FBS) supplemented with antibiotic solution containing penicillin and streptomycin (GIBCO). One million BV-173 cells were freshly marked at the start of each experiment using the Vybrant CFDA-SE Cell Tracer Kit (V12883; Invitrogen). A preliminary experiment was performed to determine the ideal CFDA-SE concentration to mark the BV-173 cells. Concentrations of 0.5, 1, 2,

and 5 μM were tested, and we chose 5 μM because of its intense and homogeneous staining of cells with cell viability similar to controls (data not shown).

Marking of BV-173 cells was performed according to the manufacturer's protocol. Briefly, the cells were incubated in prewarmed 5 μM CFDA-SE solution for 15 minutes at 37°C and then washed three times with culture medium by centrifugation. After the second wash, the cells were incubated for 30 minutes at 37°C to allow free unreacted CFDA-SE to diffuse out of the cells, before proceeding with the third wash.

Digestion of Ovarian Tissue

Human ovarian tissue was processed as previously described elsewhere (37, 41). Briefly, ovarian cortex was subjected to mechanical digestion using a tissue chopper (McIlwain Tissue Chopper; Mickle Laboratory) and incubated with 0.28 Wünsch U/mL Liberase DH (Roche Diagnostics) diluted in 10 mL of Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (Lonza, BioWhittaker) for 75 minutes in a water bath at 37°C with gentle agitation. Tissue digestion was halted by addition of 10 mL of cold PBS supplemented with 10% FBS.

Addition of Marked BV-173 Cells to the Digested Ovarian Tissue Suspension

We then added BV-173 leukemic cells marked with 5 μM CFDA-SE to the ovarian cell suspension, and the resulting mixture was centrifuged (500 rpm, 10 minutes). The number of leukemic cells added was calculated based on biopsy volume, average volume density of blood vessels in the ovarian cortex [5%, Delgado-Rosas et al. (42)], and average blood lymphoblast count of leukemia patients with tissue stored in our bank at the time of cryopreservation (3,000 cells/μL of blood), according to this following formula: 3,000,000 lymphoblasts/mL * 5% * biopsy volume (mL).

Follicle Pickup and Mechanical Purging of Contaminating Cells

The resulting pellets were resuspended in PBS supplemented with FBS, transferred to Petri dishes, and examined for follicles under a stereomicroscope (Leica, Van Hopplynus Instruments). Follicles were manually picked up using a 130-μm micropipette (Flexipet; Cook). In group 1, retrieved follicles were placed in droplets of PBS + 10% FBS without any additional treatment. In group 2, the follicles were subjected to three simple washes immediately after pickup, as enzymatically isolated follicles tend to aggregate together when left in a droplet, thereby trapping possible leukemic cells in between and making it impossible to remove them later. This involved manually pipetting and transferring the follicles to fresh droplets of PBS + 10% FBS using a 130-μm micropipette, taking special care to avoid picking up any contaminating cells. This purging step was repeated three times, and the micropipette was regularly replaced.

Detection of Leukemic Cells Using Fluorescence Microscopy

Positive controls consisted of BV-173 cells marked with CFDA-SE placed under the same lighting conditions as those in the experimental arm from the start of the experiment. After we had confirmed the persistently intense staining of all BV-173 cells in the positive control group, follicle suspensions in both groups were analyzed under a fluorescence microscope (Leica) for the presence of leukemic cells. When present, green fluorescence was visualized in these leukemic cells (λ ex/em: 495/515 nm).

PCR Analysis: Detection of the BCR-ABL Fusion Transcript in Follicle Suspensions

The aim of PCR in our samples containing a known number of leukemic cells was to test the detection limit of this technique, with a view to application in follicle suspensions from leukemia patients. We initially tested the protocol routinely used in the clinical hematology laboratory of Cliniques Univesitaires St-Luc for analysis of minimal residual disease in leukemia patients expressing the BCR-ABL fusion gene (24). Although our quantitative real-time PCR yields high sensitivity of 10^{-4} to 10^{-5}, which was an important element in the choice of this cell line, the small amount of starting material with an extraction output <100% did not allow systematic detection of the BCR-ABL fusion transcript. The detection limit was >20 leukemic cells. Therefore, before this study, a number of preliminary tests were performed, and adjustments were made to the RNA extraction and reverse transcription protocols to cater to our paucicellular samples. This lowered the detection limit to between 5 and 20 leukemic cells after quantitative real-time polymerase chain reaction (qRT-PCR).

After fluorescence analysis, follicle suspensions were transferred with a micropipette to 500 μL of TriPure isolation reagent (Roche Diagnostics) for RNA extraction. The volume of all reagents specified in the manufacturer's recommendations was divided by two due to the small quantity of total sample material, and RNA was resuspended in just 10 μL of water at the final step. All manipulations were performed manually to minimize sample loss.

Total RNA sample was used for cDNA synthesis, followed by quantitative qRT-PCR using the BCR-ABL Mbcr IS-MMR DX Kit (Ipsogen), following the manufacturer's instructions. Briefly, cDNA synthesis was performed with 10 μL of RNA to which 15 μL of reverse-transcription premix was added. After a first step at 25°C for 10 minutes, then another at 37°C for 60 minutes, reverse transcription was halted by inactivation at 85°C for 10 minutes. Quantitative RT-PCR was performed for each sample in a final volume of 25 μL, containing 10 μL cDNA for BCR-ABL detection (in duplicate) and 5 μL cDNA for ABL detection. Thermal cycling was started with an initial denaturation step at 95°C for 10 seconds, followed by 50 cycles at 95°C for 5 seconds, then 60°C for 30 seconds. Our samples and positive and negative controls were amplified in duplicate. Positive controls were commercial plasmid DNA calibrators containing target gene sequences. For negative controls, we used follicles without any BV-173 cells. Each

amplification curve was defined by its quantification cycle (Cq). The fluorescence threshold was set at the same level (above background but below plateau values) for all reactions that were compared. Results are expressed in terms of Cq for the target gene (BCR-ABL fusion transcript gene) and the housekeeping gene (Abelson: ABL).

Follicle Viability

A viability test (calcein AM/ethidium homodimer; Molecular Probes) was conducted on some of the follicles subjected to three washes as well as the control group to evaluate the impact on follicle quality. A protocol previously described by Martinez-Madrid et al. (38) was used for this purpose. Briefly, follicles were transferred to 20 μL PBS containing 2 mmol/L calcein AM and 5 mmol/L ethidium homodimer, and were incubated with the fluorescent dyes for 30 minutes at 37°C in the dark before being examined under the fluorescence microscope (Leica). Follicles were categorized based on oocyte and granulosa cell viability, as previously described by Dolmans et al. (37). The V1 and V2 represented follicles with high viability (<10% of dead granulosa cells), and V3 and V4 were follicles with poor viability (10% to 100% of dead granulosa cells or a dead oocyte).

Statistical Analyses

Statistical analysis for follicle viability was carried out using the SPSS 21 program (IBM). Two-way analysis of variance (ANOVA) was applied to compare viability between the two groups in terms of percentage of viable follicles obtained. $P<.05$ was considered statistically significant.

RESULTS

Table 1 shows the fluorescence and PCR results in both groups for all 10 experiments. In some experiments (1–3 and 8), few follicles were obtained, so they were allotted to a single group (with or without washes).

Leukemic Cell Detection by Fluorescence Microscopy

Positive controls showed intense and homogeneous staining of all leukemic cells, excluding the risk of false negatives in the experimental arm of the study (see Fig. 1A and B). In group 1 (no purging), a total of 499 follicles were recovered, among which 196 leukemic cells were detected by fluorescence microscopy (see Table 1). Figure 1C (inverted microscope) and D (fluorescence microscope) illustrates the presence of leukemic cells among retrieved follicles in this group.

In group 2, a total of 772 follicles were recovered. After pickup, follicle suspensions were subjected to three mechanical washes before leukemic cell detection. Among the 772 follicles obtained in this group, just one leukemic cell was found. In all subsequent experiments, the washes enabled us to obtain disease-free follicle suspensions. Figure 1E and F shows the absence of leukemic cells among recovered follicles in this group after the three washes.

TABLE 1

Results of leukemic cell detection among follicles in both groups.

			Group 1 (no washes)					Group 2 (3 washes)				
				Fluorescence	PCR				Fluorescence	PCR		
No.	Cortex volume (cm^3)	BV-173 cells added	No. of follicles	BV173 cells detected	Fusion transcript (Cq)	Control transcript (Cq)	Result	No. of follicles	BV173 cells detected	Fusion transcript (Cq)	Control transcript (Cq)	Result
1	0.14	21,000						50	1	ND ND	32.84	Neg.
2	0.008	1,200						6	0	ND ND	37.19	Neg.
3	0.09	13,500						9	0	ND ND	35.14	Neg.
4	0.064	9,600	100	2	ND ND	31.38	Neg.	323	0	ND ND	29.39	Neg.
5	0.2435	36,525	108	19	ND 40.99	30.58	Pos.	72	0	ND ND	32.71	Neg.
6	0.12	18,000	84	97	35.22 35.77	29.68	Pos.	70	0	ND ND	32.38	Neg.
7	0.091	13,650	49	17	ND ND	32.74	Neg.	48	0	ND ND	33.62	Neg.
8	0.118	17,700	7	1	ND ND	37.17	Neg.					
9	0.206	30,900	48	23	41.15 39.03	32.53	Pos.	112	0	ND ND	30.06	Neg.
10	0.07	10,500	103	37	38.16 36.82	30.33	Pos.	82	0	ND ND	32.45	Neg.
Total			499	196				772	1			

Note: In group 1 (no purging), between 1 and 97 leukemic cells were found to contaminate the different follicle suspensions. Polymerase chain reaction (PCR) for the BCR-ABL fusion transcript was positive in four cases and negative in three cases. The detection limit was approximately 19 BV-173 cells. In group 2, after purging, just one leukemic cell was detected (in the very first experiment) among the 772 follicles by fluorescence whereas PCR was negative in all cases. Cq = quantification cycle; Neg. = negative; ND = not detected; POS. = positive.

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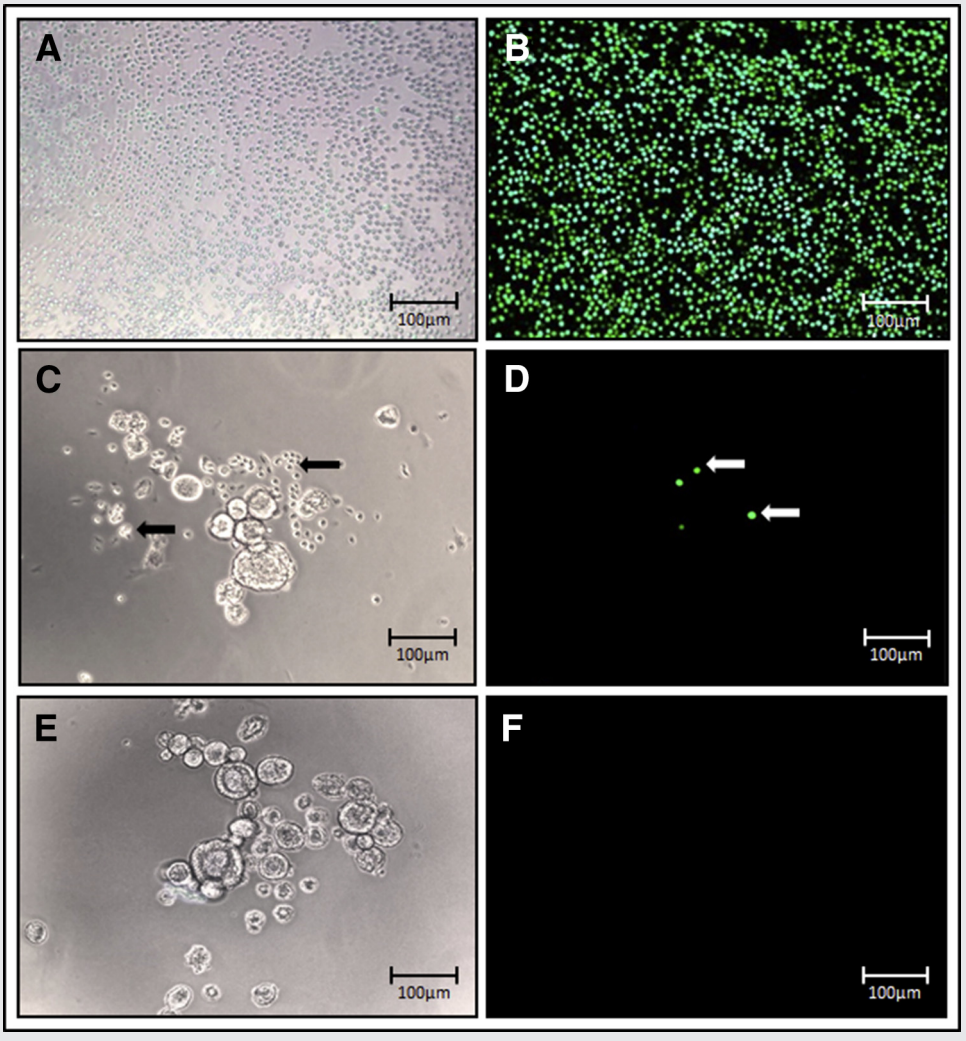
FIGURE 1



Positive controls and follicle suspensions analyzed under an inverted microscope (original magnification: ×100) on the left and a fluorescence microscope on the right. (**A** and **B**) Marked BV-173 leukemic cells used as positive controls. (**C**) Follicles obtained by the standard pickup technique without purging. Note the presence of small ovarian cells along with the follicles (*black arrows*). (**D**) Analysis of the same suspensions under a fluorescence microscope (*green filter*) reveals contamination of follicle suspensions by leukemic cells (*white arrows*). (**E**) Follicle suspension obtained after mechanical purging. Small ovarian cells have been eliminated. (**F**) No leukemic cells can be seen under the fluorescence microscope.

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Analysis of the wash droplets as well as follicle suspensions before washing also was performed under inverted and fluorescence microscopes. Follicle suspensions in group 2, examined before the washes, contained between 2 and 83 leukemic cells. Evaluation of the different wash droplets revealed that the large majority of leukemic cells in follicle suspensions (93.06%) were eliminated in the first wash, 5.56% in the second, and the remaining 1.38% in the third (Fig. 2). All cells were recovered, and there was no loss during manipulation.

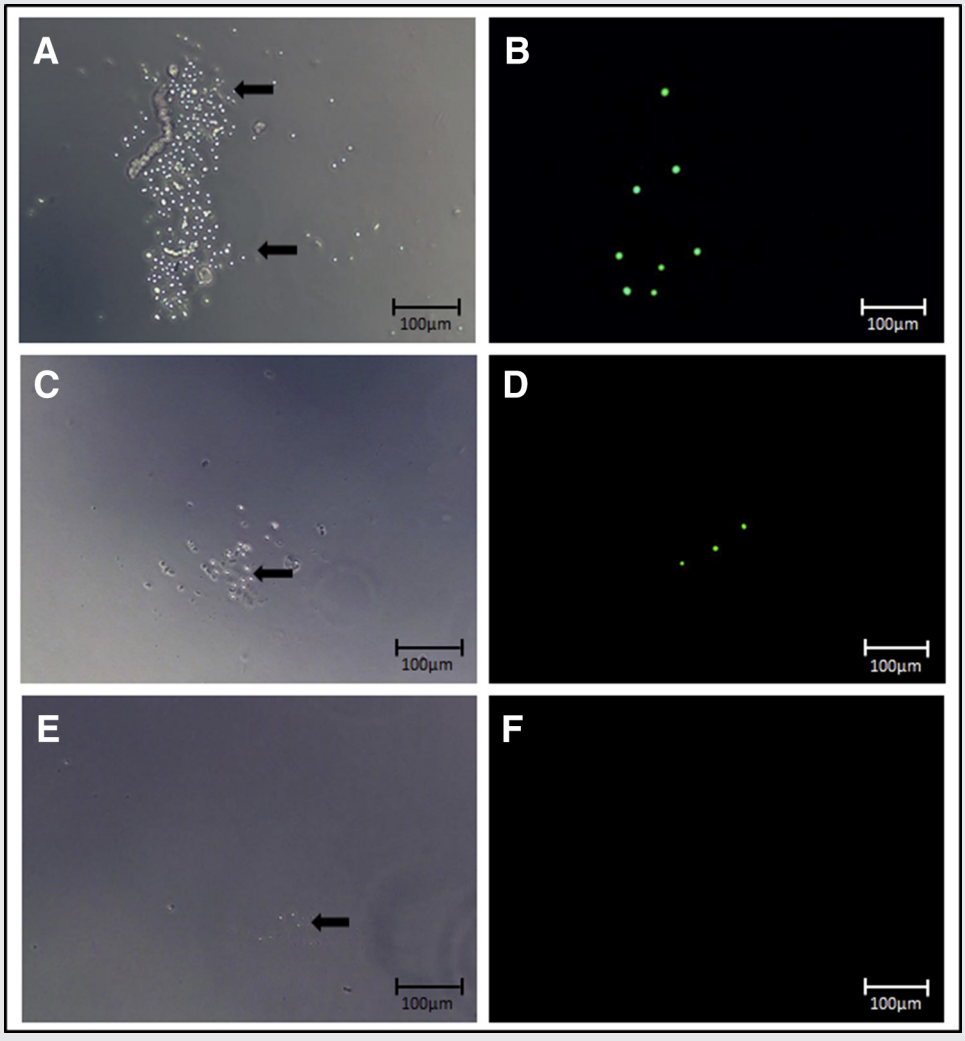
PCR Analysis

For follicle suspensions in group 1, the BCR-ABL fusion transcript was detected in four out of seven samples. The detection limit using our conventional extraction and PCR protocols appears to be around 20 leukemic cells, as samples (in duplicate) containing 1, 2, and 17 BV-173 cells at fluorescence microscopy remained negative at PCR whereas the sample containing 19 cells was positive once (with a cycle threshold of 40.99) and negative once. In group 2, when follicles were washed three times, there was no sign of the BCR-ABL fusion gene in any of the nine follicle samples.

Follicle Viability

The viability of the 85 follicles obtained after the three follicle washes and the 46 follicles recovered immediately after pickup (without washing) was evaluated using a calcein AM/ethidium homodimer viability assay (Supplemental Fig. 2, available online). Group V1 represents follicles with

FIGURE 2



Analysis of remaining medium droplets after mechanical purging under (**A**, **C**, and **E**) inverted and (**B**, **D**, and **F**) fluorescence microscope (original magnification: ×100), showing progressive elimination of contaminating ovarian and leukemic cells with the three washes. The large majority of contaminating cells (*black arrow*) are eliminated during the first manual pipetting (**A** and **B**), a further few during the second purge (**C** and **D**), and the last remaining cells, if present, during the third step (**E** and **F**). This confirms the need for three-step purging.

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a viable oocyte and granulosa cells, and V2 those with less than 10% of dead granulosa cells (37). The results are presented in Table 2. Follicles in both groups showed very high viability: 96.4% in the group after three washes (87% V1 and 9.4% V2) versus 95.6% in the control group (82.6% V1 and 13% V2). There was no statistically significant difference in viability between the two groups ($P=.9$).

DISCUSSION

Various studies have shown that ovarian tissue from patients with leukemia may harbor leukemic cells (23–25). In one study, transplantation of ovarian tissue from patients with acute leukemia to immunodeficient mice was found to reintroduce the disease in 5 out of 12 cases (24). The concentration of malignant cells is likely to be low in such cases, casting doubt upon their viability and potential to retransmit the disease after cryopreservation and thawing when the ovarian tissue is harvested after chemotherapy. Indeed, results from a Danish study using a murine model showed that cryopreserved ovarian tissue from patients in complete remission did not appear to contain any viable malignant cells. Although the PCR results were positive, none of the grafted mice developed the disease (43). However, the risk of disease retransmission through the graft cannot yet be excluded, and further studies are required before transplantation procedures can be safely performed (9, 44, 45).

For patients at risk of ovarian involvement, one option to restore fertility could be the grafting of isolated follicles enzymatically purified from frozen-thawed ovarian tissue (37, 41). It is nevertheless vital to ensure that these follicles are

TABLE 2

Results of follicle viability.

	Group 1: No washes (n = 46)				Group 2: 3 washes (n = 85)			
No.	V1	V2	V3	V4	V1	V2	V3	V4
1	13	2	0	0	18	0	1	0
2	11	1	0	1	26	1	1	0
3	5	1	0	0	14	4	1	0
4	9	2	0	1	16	3	0	0
Total	38	6	0	2	74	8	3	0

Note: Follicles in both groups showed very high viability: 96.4% in the group after the washes (87% V1 and 9.4% V2) versus 95.6% in the control group (82.6% V1 and 13% V2).

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completely free of any malignant cells before transplantation can be contemplated. In leukemia patients, for example, if leukemic cells are present in ovarian tissue, they could also potentially be found in the ovarian cell suspension after enzymatic digestion.

Because cryopreserved ovarian tissue from leukemia patients is scarce and precious, in this preliminary study the leukemic cells were artificially added to digested ovarian tissue suspensions obtained from healthy patients. Very few data are available on the number of leukemic cells that may be present in ovarian tissue of patients with leukemia. In a study using eight-color flow cytometry, Amiot et al. (26) detected minimal disseminated disease in a patient with T-cell acute lymphoblastic leukemia at a level of 2.7×10^{-4} (365 minimal residual disease-positive events among 1.36×10^{6} total alive nucleated events). In other studies, the number of PCR cycles required to amplify the gene appeared to be indicative of a low concentration of malignant cells (23, 24, 43). For our study, the number of leukemic cells added to our suspensions was calculated based on biopsy volume, average volume density of blood vessels in ovarian cortex [5% (42)], and average blood lymphoblast count of leukemia patients with their tissue stored in our bank at the time of cryopreservation (3,000 cells/μL of blood), which represents the number of cells that could theoretically be present in cryopreserved cortex.

This study allowed us to evaluate the accuracy of follicle isolation and pickup when ovarian tissue is artificially contaminated with malignant cells. Our results show that in cases of relatively extensive contamination, there is a risk that a few cells could be retrieved along with isolated follicles during follicle pickup. Indeed, during manual pipetting of follicles, some stromal cells can be inadvertently picked up as well. Leukemic cells are around the same size or slightly bigger ($\sim$10–12 μm), and they resemble ovarian stromal cells under the stereomicroscope, making it impossible to distinguish between them during pickup (see Figs. 1 and 2).

After follicle pickup between 1 and 97 leukemic cells were found to contaminate the follicle suspensions of patients. The number of human leukemic cells required to reintroduce the disease is currently unknown, and the few available data from rodent models are contradictory. In rats, as few as 20 leukemic cells (rat T-cell leukemia cell line isolated from spontaneously leukemic rats) injected into the testis were capable of inducing leukemia in recipients (46). On the other hand, another group demonstrated that more than 1×10^{6} Jurkat cells (human T-cell leukemia cell line) were required for tumor development when injected subcutaneously into nude mice (47). These discrepant results may be partly explained by the host, aggressiveness of the different leukemia cell lines used (47) and different grafting sites.

Following the present study, we turned our attention to developing an animal model to test the capacity of a few leukemic cells to induce the disease after long-term xenografting to immunodeficient mice. Ten and 100 leukemic cells (based on the 1 to 97 cells found in follicle suspensions, before washing, in group 1 of this study) were embedded along with other ovarian cells in a fibrin matrix and grafted to a created peritoneal pocket in SCID mice. We found that as many as 100 BV-173 cells grafted in a fibrin matrix were not sufficient to induce leukemia (48). However, with a view to clinical application, it is imperative to achieve complete elimination of malignant cells from the follicle suspension.

Marking our leukemic cells before addition to ovarian suspensions allowed use of fluorescence microscopy, so we were able to detect cells in follicle suspensions with 100% accuracy in this model. Our simple procedure, which involved washing the follicles three times by manually pipetting and transferring them to fresh medium droplets allowed successful elimination of contaminating leukemic cells. Just one leukemic cell was detected after the washes in the very first experiment, which may be considered part of the learning curve. These favorable results are all the more reassuring given the relatively large number of leukemic cells added to the suspensions.

It is also important to ensure that follicles, which are complex and fragile structures, are not damaged due to treatment. Follicle viability was tested after the three washes using the calcein AM/ethidium homodimer viability test and showed very good viability, with no difference compared with controls. The next step will be to confirm this by culture and xenotransplantation of washed follicles.

Techniques such as fluorescence-activated cell sorting (FACS) (49–52) and use of bispecific antibodies (53) have also been tested by various groups for the elimination of cancer cells from spermatogonial and ovarian cell suspensions, with suboptimal or divergent results. For our application, however, the size difference between follicles on the one hand, and leukemic or ovarian stromal cells on the other, makes it difficult to use FACS to reliably separate cell populations. Moreover, enzymatic treatment could alter the immunophenotype of cells. Possible aggregation of cancerous or noncancerous cells may decrease FACS efficiency and thus the safety of this cell sorting method (50). The manipulations and time involved could also lead to follicle loss and decreased viability.

One of the limitations of our study is that interactions between malignant cells and follicles in naturally invaded human ovaries could well differ from those in our model, where leukemic cells were added to the digested ovarian suspension. These results will therefore need to be corroborated using ovarian tissue from leukemia patients before clinical

application may be contemplated. To do so, it is vital to develop techniques capable of detecting very small numbers of cells in follicle suspensions, as fluorescent marking of leukemic cells used in the present study is not an option for malignant cell detection in follicle suspensions from leukemia patients.

Two kinds of markers are currently used to identify minimal residual disease in patients with leukemia: genetic markers, which can be detected by PCR, or multiple immunophenotypic markers, detected by flow cytometry. Flow cytometry was not suitable for the follicle suspensions due to the very small number of cells in our samples and the size difference between follicles and leukemic cells. Furthermore, the threshold for samples to qualify as positive at flow cytometry is at least 20 positive elements. Multiple immunofluorescence protocols were tested (data not shown) but with systematic loss of follicles and leukemic cells during staining (54). In our present study, PCR was performed on follicle suspensions to test the detection limit of this technique. Our qRT-PCR for detection of the BCR-ABL fusion transcript has very high sensitivity of 10^{-4} to 10^{-5} (54). Before this study, a number of preliminary tests were conducted and, by adjusting the protocol routinely applied in our clinical hematology laboratory for minimal residual disease detection in leukemia patients (24), we were able to detect between 5 and 20 leukemic cells in our paucicellular samples after PCR. We tried replacing our PCR technique with the new nonofluidic digital droplet PCR system (Bio-Rad Laboratories); however, because the major problem was not the sensitivity of the technique but the material obtained after RNA extraction, it did not have any discernible impact. With a view to clinical application, we need to ensure maximum accuracy to detect a minimum number of malignant cells. The problem encountered in our study was the very small number of cells in our samples (as few as six follicles and very few leukemic cells) and the extraction output, which was not 100%.

The key to lowering the detection limit lies in increasing the quantity of RNA material in our samples before PCR. To this end, we recently tested a kit (RNeasy Plus Micro Kit, cat no: 74034; QIAgen) specially designed for isolation of RNA from a very small number of cells. Increasing numbers (2, 5, 10, 20, and 80) of leukemic cells were manually added to follicle suspensions using a micropipette. With this extraction technique followed by reverse transcription and qRT-PCR analysis of whole sample, we were able to lower our detection limit to two to five cells (data not shown), which is as accurate as possible for now. Indeed, we are not aware of any other technique that offers better detection at present.

In conclusion, this study evaluates a follicle isolation technique to obtain disease-free follicle suspensions when malignant cells are present in ovarian cell suspensions. It shows that in cases of relatively extensive contamination of ovarian tissue by malignant cells some of these cells may be found in the follicle suspension after pickup. However, three simple washes proved sufficient to effectively eliminate the contaminating cells inadvertently retrieved along with follicles while at the same time maintaining good follicle viability. These results will now be corroborated using ovarian tissue from leukemia patients.

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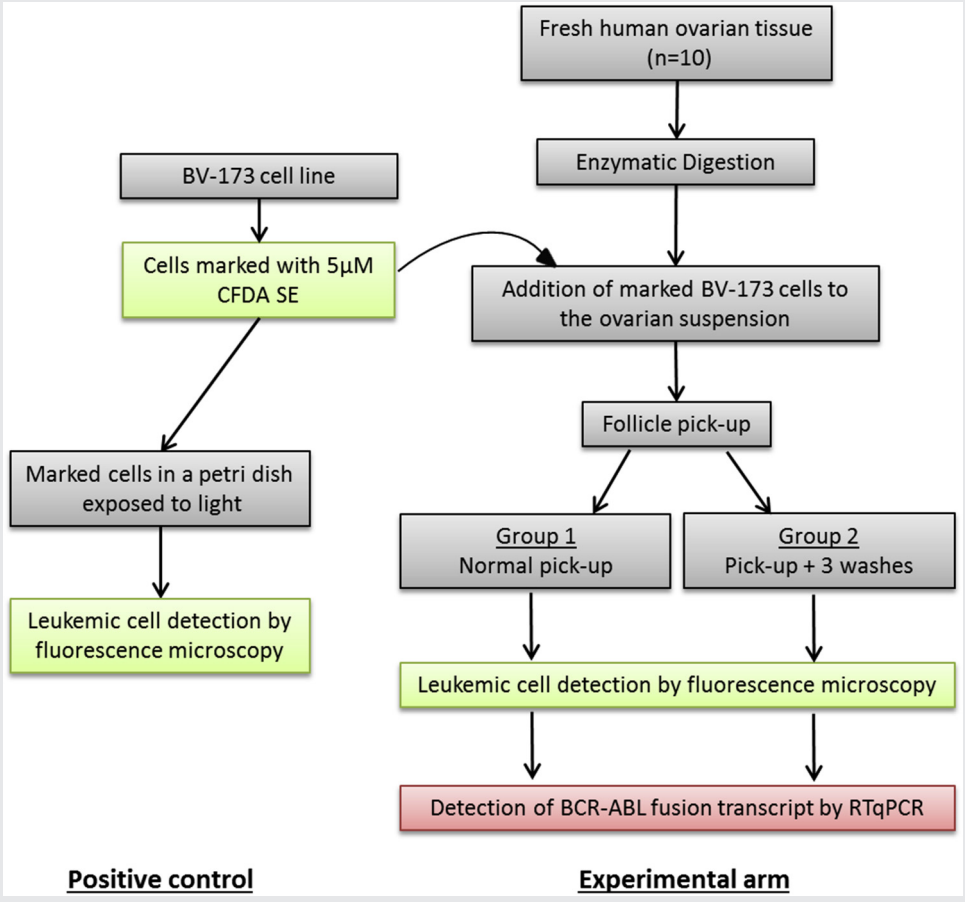
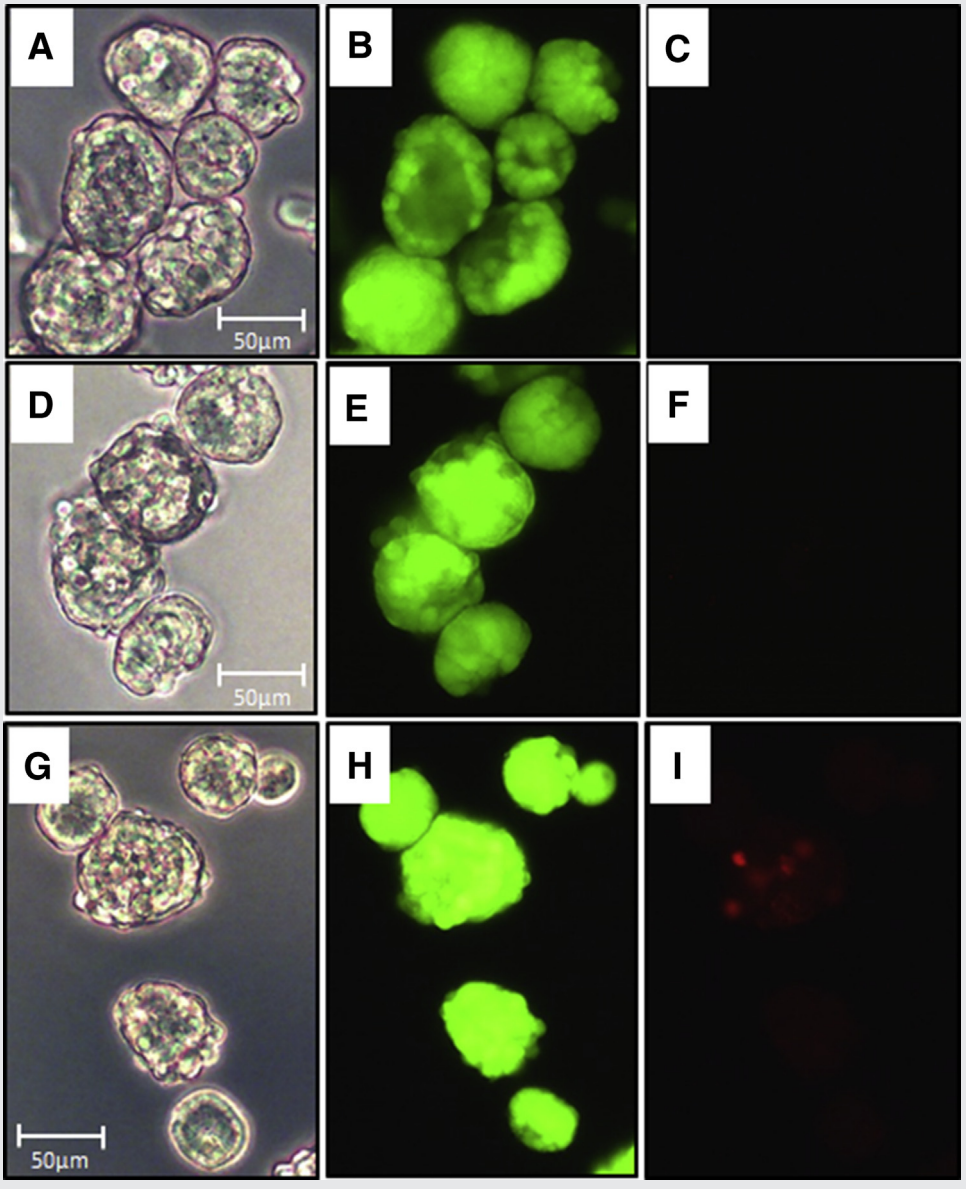
SUPPLEMENTAL FIGURE 1



Study design.

Soares. Safe follicle isolation for cancer patients. Fertil Steril 2015.

SUPPLEMENTAL FIGURE 2



Viability test of isolated human ovarian follicles after three-step purging (original magnification: ×200). (**A**, **D**, and **G**) Follicles observed without a filter. A live/dead kit was used for viability testing and follicles were analyzed under the fluorescence microscope. (**B**, **E**, and **H**) *Green* filter: viable cells are stained with calcein AM and emit *green* fluorescent light. (**C**, **F**, and **I**) *Red* filter: dead cells are stained with ethidium homodimer-1 and emit *red* light.

Soares. Safe follicle isolation for cancer patients. Fertil Steril 2015.