Low doses of alkylating agents do not harm human ovarian tissue destined for cryopreservation

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Objective: To investigate the impact of nongonadotoxic doses of alkylating agents on human ovarian cortex.
Design: Retrospective study.
Setting: Academic research center.
Patients: Biopsies from 78 patients who had undergone ovarian tissue cryopreservation were retrieved and analyzed. Among them, 42 had previously been treated with chemotherapy (alkylating agents, dose <3,400 mg/m^2), making up the chemotherapy group, whereas 36 had not been given any chemotherapy, constituting the control group.
Mean Outcome Measures: Follicle count and classification, morphology study, immunostaining for apoptosis (cleaved caspase-3), immunostaining for activation (phospho-Akt), fibrosis (Masson's trichrome), and vascularization (von Willebrand factor and smooth muscle actin).
Results: In the prepubertal group, 271 follicles/mm^3 were detected in control patients and 501 follicles/mm^3 in chemotherapy-exposed subjects. In the adult group, 4,916 follicles/mm^3 were found in control patients and 6,570 follicles/mm^3 in chemotherapy-exposed patients. No difference in follicle density was observed between the 2 groups in any age category. Neither did we encounter any significant difference in follicle viability according to chemotherapy exposure or age. Proportions of nongrowing follicles were >76% in all age groups, irrespective of chemotherapy exposure, and higher, although not significantly, in the chemotherapy group compared with the control group. There were significantly fewer secondary follicles in the adult chemotherapy group than in the adult control group. Concerning apoptosis, no significant difference was observed between control and chemotherapy subjects in any age groups. Numbers of activated follicles were systematically higher in all age categories in the chemotherapy group than the control group. Areas of atypical follicles were noted in 4 out of 14 prepubertal patients in the chemotherapy group. In these areas, follicle density was 84,570 ± 8,837 follicles/mm^3 and all follicles appeared nonviable but showed no sign of apoptosis.
Conclusion: Low-dose chemotherapy had no major impact on ovarian tissue, suggesting that ovarian tissue exposed to some chemotherapy before cryopreservation is comparable with ovarian tissue free of any chemotherapy, as clinically demonstrated by high pregnancy rates after ovarian tissue transplantation in women exposed to chemotherapy. Previous chemotherapy should therefore no longer be a contraindication to ovarian tissue cryopreservation. (Fertil Steril® 2024;■:■–■. ©2024 by American Society for Reproductive Medicine.)

Key Words: Ovarian tissue, chemotherapy, alkylating agents, ovarian tissue cryopreservation, follicle density, activation, apoptosis

In recent years, remarkable strides have been made in cancer management, looking to improve patient survival rates and outcomes. However, alongside therapeutic advances, these treatments also have adverse consequences, giving rise to major concerns about long-term effects of cancer therapies. One notable off-target outcome is iatrogenic premature ovarian insufficiency (POI), which curtails both reproductive capacity and systemic endocrine function (1–3). This has

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profound repercussions on women's health and poses a significant challenge in clinical practice, especially in young patients expecting a typical reproductive lifespan. Indeed, premature menopause can dramatically impact quality of life in these individuals (4).

A number of fertility preservation options are available, like immature and mature oocyte cryopreservation, or ovarian tissue cryopreservation (OTC) followed by transplantation (OTT) (1). The latter is the only alternative for prepubertal girls and patients whose chemotherapy cannot be delayed. (1, 5) There is now sufficient evidence to support the feasibility and efficacy of OTC and OTT for both fertility preservation and restoration of ovarian endocrine function (1, 6). Nevertheless, strict selection criteria are essential, which is why the Edinburgh criteria were introduced in 2015. They prioritize factors such as age under 35 years, a realistic 5-year survival outlook, and at least a 50% risk of POI. Previous chemotherapy is considered a contraindication to OTC (7), but recent studies report similar pregnancy rates in individuals undergoing OTC after chemotherapy to those without prior chemotherapy, suggesting that chemotherapy should not be considered a contraindication (6, 8, 9). Indeed, in these studies, live birth rates were 30%–40% after OTT in patients who had undergone OTC after exposure to some chemotherapy.

Chemotherapy affects the ovarian reserve through multiple pathways, like apoptosis in growing follicles (10), overactivation and growth of primordial follicles (11), and stromal and vascular damage (12, 13). The goal of our study was therefore to investigate any changes induced by chemotherapy in ovarian cortex from women exposed to these drugs and evaluate their impact on follicle density and quality by comparing the findings with cortex from women who never had chemotherapy before OTC.

MATERIALS AND METHODS

Experimental design

Use of human tissue was authorized by the Ethics Committee of Cliniques Universitaires Saint-Luc under reference 2023/16MAI/228. Follicle density was assessed by hematoxylin and eosin staining and follicle characteristics by immunohistochemistry targeting phospho-Akt (p-Akt) for follicle activation and cleaved caspase-3 for apoptotic follicles. The stromal environment was evaluated by Masson's trichrome staining for the presence of fibrosis, as well as immunofluorescence co-staining to indicate vascular maturity.

Patients

Seventy-eight formalin-fixed paraffin-embedded blocks of ovarian tissue samples were obtained from patients undergoing OTC for oncological reasons: 42 from women previously treated with chemotherapy (chemotherapy group) (obtained from the Biobank of the Anatomopathology Department of Pitié-Salpêtrière Hospital in Paris) and 36 from chemotherapy-free women serving as controls (control group) (acquired from the Biobank of the Anatomopathology Department of Cliniques Universitaires Saint-Luc). Subjects were matched for age and pathology to mitigate pathology-linked bias. We categorized our patients into 4 groups determined by age: prepubertal (3–8 years old); peripubertal (9–14 years old); postpubertal (15–25 years old); and adult (>25 years old) (Table 1). The peripubertal group was designed to include patients whose pubertal status was uncertain due to their borderline age.

In the chemotherapy group, maximum doses of alkylating agents were 60,000 mg/m^2 ifosfamide and 3,400 mg/m^2 cyclophosphamide. These values do not exceed the threshold for high doses of alkylating agents set at 60,000 mg/m^2 ifosfamide and 7,500 mg/m^2 cyclophosphamide (14). Moreover, OTC was performed in all cases less than 3 months after chemotherapy exposure. In addition to other nongonadotoxic drugs given as part of the established treatment protocols, 14 subjects received ifosfamide, 26 cyclophosphamide, and 2 both drugs (total dose lower than the gonadotoxic limit). In the control group, none of the patients showed any signs of malignancy in their ovarian cortex, as determined by the pathologist at the time of OTC.

Subgroup analysis

In prepubertal patients in the chemotherapy group, atypical areas were observed in 4 subjects (3 neuroblastomas and 1 acute lymphoblastic leukemia). A substudy was conducted with these patients' data, analyzing their results separately from the rest of the prepubertal chemotherapy group.

Histology

Ovarian fragments were fixed in 4% formaldehyde immediately after surgery and incorporated inside paraffin blocks for long-term storage and in-depth anatomopathology analysis of the preserved tissue. Indeed, pathology assessment is crucial to establish the presence of malignant infiltration, but also follicle density, with a view to future transplantation decisions. In our institutions, biologists are highly trained and well aware of the importance of these thorough inspections of fixed tissue. They therefore allocate representative specimens of cryopreserved tissue for fixation with full knowledge of the facts.

Five-micrometer sections were obtained from each block, and up to 3 nonconsecutive sections (50 μm apart) from each sample were mounted on either blue or white glass slides to perform histological and immunohistochemical staining on stored ovarian cortex. After staining, all slides were digitized using the SCAN II Pannoramic slide scanner (3DHistech, Budapest, Hungary) and analyzed by CaseViewer v2.4 (3DHistech, Budapest, Hungary).

Follicle density and classification

Follicle density and classification were evaluated as previously described by our laboratory (15) (Supplemental 1, available online). Follicle classification and counting were determined according to established criteria (16), as follows: primordial: a single layer of flattened granulosa cells (GCs) surrounding the oocyte (Fig. 1A); intermediate: at least 2 cuboidal GCs surrounding the oocyte; primary: a complete

TABLE 1

Patient characteristics.

Age group	Chemotherapy (n = 42)	Control (n = 36)
Prepubertal (3–8 y)	5.33 ± 1.77, ALL (n = 4), neuroblastoma (n = 5), sarcoma (n = 3)	5.33 ± 1.58, ALL (n = 2), neuroblastoma (n = 3), sarcoma (n = 4)
Peripubertal (9–14 y)	12 ± 1.22, ALL (n = 3), sarcoma (n = 6)	11 ± 1.85, ALL (n = 3), sarcoma (n = 5)
Postpubertal (15–25 y)	18.67 ± 3.11, ALL (n = 5), HL (n = 5), sarcoma (n = 5)	17.8 ± 2.57, ALL (n = 5), HL (n = 5), sarcoma (n = 5)
Adult (>25 y)	28.33 ± 1.75, ALL (n = 2), HL (n = 3), sarcoma (n = 1)	30 ± 3.92 HL (n = 4)

Mean age ± SD. Seventy-eight formalin-fixed paraffin-embedded blocks of ovarian tissue were obtained from patients undergoing OTC for oncological reasons: 42 from women previously treated with chemotherapy (chemotherapy group) (obtained from the Biobank of the Pitié-Salpêtrière Hospital in Paris) and 36 from chemotherapy-free women serving as controls (control group) (acquired from the Biobank of the Anatomopathology Department of Cliniques Universitaires Saint-Luc). Subjects were matched for age and pathology and then divided into 4 age groups: prepubertal (3–8 y); peripubertal (9–14 y); postpubertal (15–25 y); and adult (>25 y).
ALL = acute lymphoblastic leukemia; HL = Hodgkin lymphoma; OTC = ovarian tissue cryopreservation.

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layer of cuboidal GCs surrounding the oocyte (Fig. 1B); secondary: 2 or more layers of cuboidal GCs with no antrum surrounding the oocyte (Fig. 1C); antral: several layers of cuboidal GCs, an antrum and cumulus surrounding the oocyte; and atretic: irregular basement membrane, eosinophilic cytoplasm, and pyknotic nuclei. Nongrowing follicles (NGFs) included primordial and intermediate types, and growing follicles primary, secondary and antral categories. Follicles were considered abnormal if they contained 2 oocytes or 2 nuclei, and if vacuoles were observed in GCs or the oocyte without any sign of atresia (17) (Fig. 1A).

Follicle apoptosis

Follicle apoptosis was immunodetected with the cleaved caspase-3 primary antibody (9661S, Cell Signaling) according to a protocol already published by our laboratory (15) (Supplemental 2). A follicle was considered positive if the oocyte or >50% of GCs were stained for caspase-3 (18).

Follicle activation

Follicle activation was immunodetected with the p-Akt primary antibody (4060P, Cell Signaling) also following a protocol previously published by our laboratory (19) with image analysis performed as already reported (20) (Supplemental 3). Activation rates per patient were calculated on the basis of the average percentage of stained area in all NGFs on each slide.

Stromal fibrosis

Stromal fibrosis was assessed using Masson's green trichrome staining to identify collagen-rich (fibrotic) connective tissue (Supplemental 4).

Vascularization

Vascularization in ovarian cortex was evaluated following a protocol well established in our laboratory (21) using double immunofluorescence staining with anti-human von Willebrand factor antibody (A0082, Agilent) for all blood vessels, and anti-smooth muscle actin polyclonal antibody (M085101, Agilent) for mature blood vessels (Supplemental 5).

Statistical analysis

Data were statistically analyzed using GraphPad Prism software (v8.0.1, Boston, MA). Normality and lognormality tests were performed to check for adherence to normal (Gaussian) distribution. Multiple t-tests were then conducted to assess any statistically significant differences between groups, with significance set at $P<.05$. Appropriate tests were applied when distribution was not normal. Finally, descriptive statistics were used to determine means, SDs, medians, and ranges.

RESULTS

Follicle count

In the control group, median (range) follicle densities (Fig. 2A) were 4,216 (1,298–8,259) foll/mm^3 in the prepubertal group, 2,309 (667–11,313) foll/mm^3 in the peripubertal group, 632 (182–2,797) foll/mm^3 in the postpubertal group, and 271 (120–1,888) foll/mm^3 in the adult group.

In the chemotherapy group, median (range) of follicle densities were 6,570 (575–20,136) foll/mm^3 in the prepubertal group, 3,738 (477–12,352) foll/mm^3 in the peripubertal group, 981 (0.05–4,381) foll/mm^3 in the postpubertal group, and 501 (152–1,664) foll/mm^3 in the adult group.

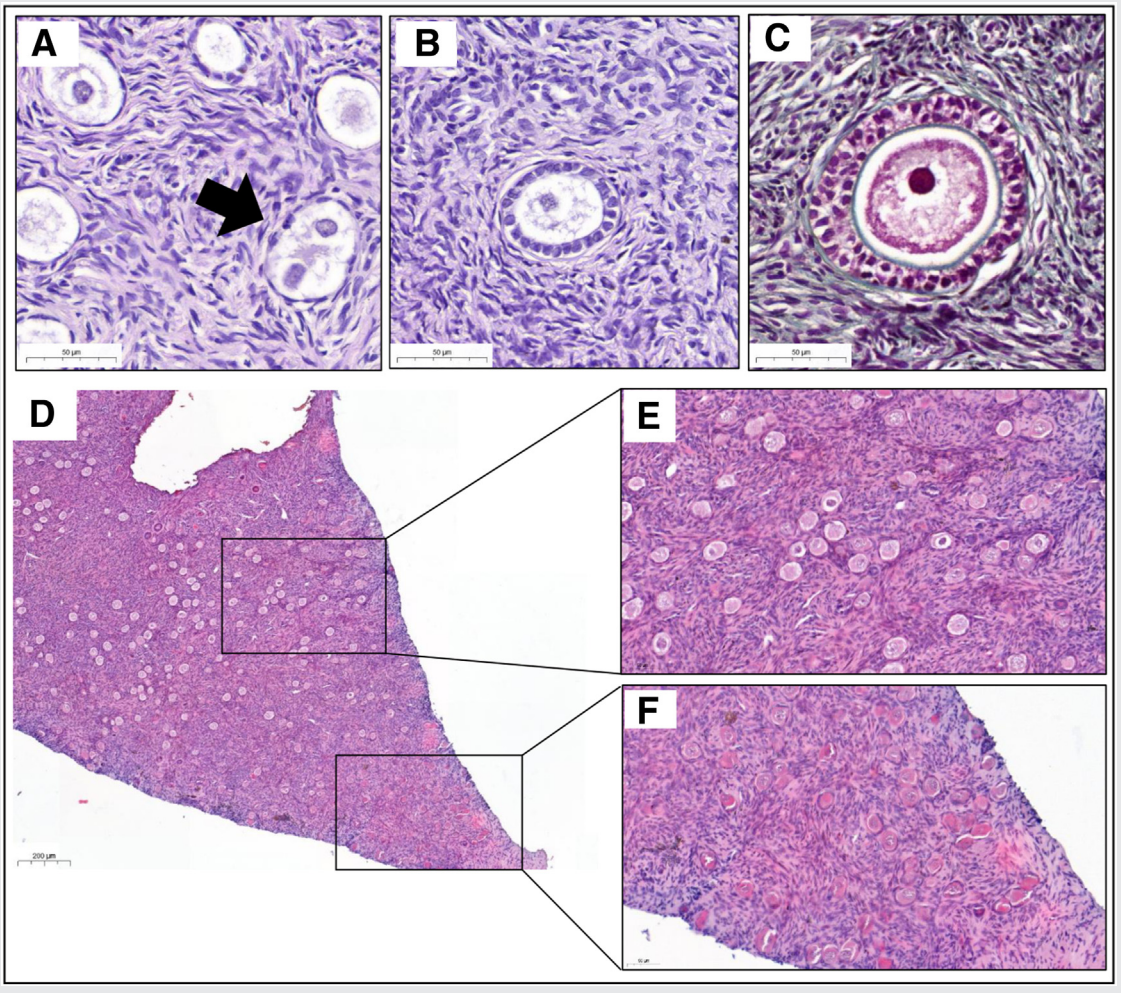
Analysis of follicle density did not reveal any statistically significant difference between subjects in the chemotherapy group and control group in any age category. However, they both showed a decrease in follicle density according to age (Fig. 2A).

Follicle morphology

Viable follicles. We investigated proportions of viable follicles out of all follicles. In the control group, means ± SDs were 74.2% ± 16.2% in prepubertal patients, 69.8% ± 16.3% in peripubertal patients, 75.6% ± 20.6% in postpubertal patients, and 98.4% ± 2.8% in adult patients. In the chemotherapy group, means ± SDs were 69.2% ± 21.8% in prepubertal patients, 73.5% ± 19.9% in peripubertal patients, 73.7% ± 12.3% in postpubertal patients, and 72.3% ± 15.8% in adult patients. No significant difference was noted in follicle viability according to chemotherapy exposure or age.

Abnormal follicles. Abnormal follicles were defined as either double follicles, with 2 nuclei or 2 oocytes in the same GC layer, or vacuolized if vacuoles were visible without signs of

FIGURE 1



Follicle morphology. (**A**) Abnormal follicle (arrow) in H&E staining: one follicle containing 2 oocytes surrounded by normal viable NGFs: one single layer of flat GCs around the oocyte. (**B**) Normal viable primary follicle in H&E staining: one single layer of cuboidal GCs around the oocyte. (**C**) Normal viable secondary follicle in Masson's trichrome staining: at least 2 layers of cuboidal GCs around the oocyte; collagen fibers are colored in green. (**D**) Histology section of ovarian cortex from a prepubertal patient with ALL exposed to chemotherapy before OTC. (**E**) Normal cortex surrounding an area of atypical cortex. (**F**) Atypical cortex area: ill-shaped atretic follicles with an intensely eosinophilic cytoplasm. ALL = acute lymphoblastic leukemia; GC = granulosa cell; H&E = hematoxylin and eosin; NGF = nongrowing follicle; OTC = ovarian tissue cryopreservation.

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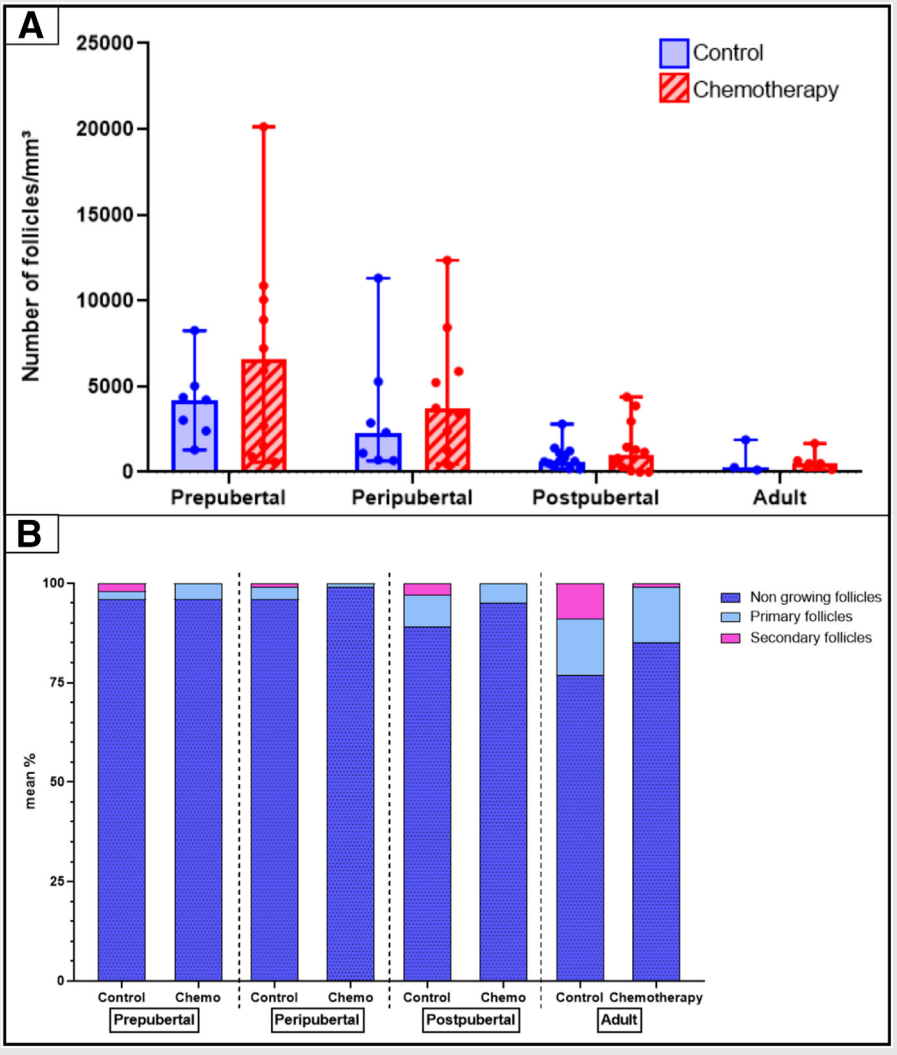
atresia (Fig. 1A). Proportions of abnormal follicles were calculated out of the number of viable-looking follicles. In the control group, medians (range) were 5.6% (0–32.2) in prepubertal patients, 7.9% (2.9–22.8) in peripubertal patients, 5.9% (0–22.2) in postpubertal patients, and 0% (0–25) in adult patients. In the chemotherapy group, medians (range) were 5.14% (0–32.9) in prepubertal patients, 2.4% (0–36.4) in peripubertal patients, 1.9% (0–5.4) in postpubertal patients, and 2.6% (0–6.0) in adult patients. There was a drastic drop in rates of abnormal follicles in all adult groups, but no significant difference between chemotherapy and control subjects.

Nongrowing follicles. NGF proportions were calculated out of the population of normal viable follicles. In the control group, means ± SDs were 96.0% ± 5.6% in prepubertal patients, 96.5% ± 6.2% in peripubertal patients, 89.5% ± 14.1% in postpubertal patients, and 77.0% ± 23.6% in adult patients. In the chemotherapy group, these figures were respectively 95.5% ± 6.8%, 99.2% ± 1.1%, 94.5% ± 6.8%, and 84.9% ± 11.5% (Fig. 2B). Proportions of NGFs out of all viable follicles were >76% in all age groups, whether exposed to chemotherapy or not. These proportions were nevertheless higher, even if not significantly so, in the chemotherapy group compared with the control group.

We compared the percentage of primordial follicles out of total NGFs in both groups. In controls, means ± SDs of primordial follicles/NGFs were 81.88% ± 15.79% in the prepubertal group, 80.55% ± 14.22% in the peripubertal group,

FIGURE 2



Follicle density and classification. (**A**) Follicle density: median ± range. There was a decrease in numbers of follicles/mm^3 according to age, but no significant difference between chemotherapy-exposed patients and control subjects. (**B**) Follicle proportions: % of NGFs, primary follicles and secondary follicles out of normal viable follicles. There was no difference in NGF proportions between chemotherapy and control patients in any age groups, nor indeed in terms of primary follicles in the same categories. However, no secondary follicles were found in the postpubertal chemotherapy group and there were significantly fewer secondary follicles in the adult chemotherapy group than the adult control group (P=.009). NGF = nongrowing follicle.

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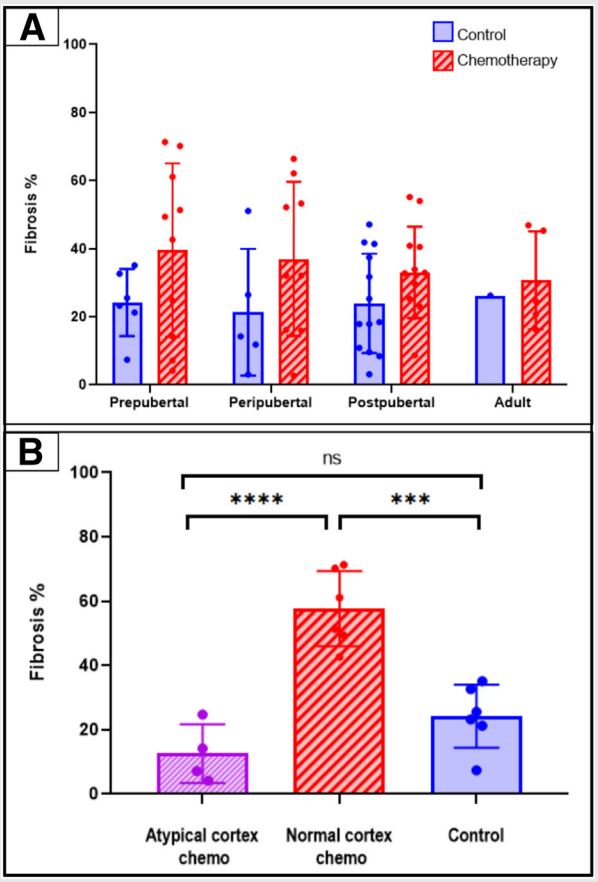
77.23% ± 12.54% in the postpubertal group and 82.94% ± 16.27% in the adult group. In chemotherapy subjects, these values were respectively 72.77% ± 10.31%, 63.51% ± 21.96%, 63.21% ± 12.54%, and 58.54% ± 10.90%. No significant difference was observed between groups.

Growing follicles: primary and secondary follicles. Proportions of primary and secondary follicles were also calculated out of the population of normal viable follicles. In the control group, medians (range) of primary follicle proportions were 1.9% (0–5.1) in the prepubertal group, 0% (0–8.6) in the peripubertal group, 0% (0–33.3) in the postpubertal group, and 12.5% (3.9–25.0) in the adult group. In the chemotherapy group, these medians were respectively 1.4% (0–20.8), 0% (0–2.8), 4.5% (0–25.0), and 12.9% (2.7–26.7).

For secondary follicles, medians (range) in controls were 0% (0–11.54) in the prepubertal group, 0% (0–8.6) in the peripubertal group, 0% (0–15.4) in the postpubertal group, and 2.6% (0–25.0) in the adult group. In the chemotherapy group, medians (range) of secondary follicle proportions were respectively of 0% (0–4.4), 0% (0–0), 0% (0–1.7), and 0% (0–1.9).

As shown in Figure 2B, there was an increase in growing follicles according to age. No secondary follicles were found in the postpubertal chemotherapy group and there were

FIGURE 3



Fibrosis rates. (**A**) In all age groups, fibrosis was systematically more substantial in the chemotherapy group than in controls, although it was not significant. In the control group, means ± SDs of fibrotic area out of total area were 24.2% ± 9.8% in prepubertal patients, 21.3% ± 18.6% in peripubertal patients, 23.9% ± 14.6% in postpubertal patients, and 26.2% ± 0% in postpubertal adults. In the chemotherapy group, means ± SDs were, respectively, 39.6% ± 25.4%, 37.0% ± 22.6%, 33.1% ± 13.4%, and 30.7% ± 14.3%. (**B**) Areas of fibrosis observed in the atypical cortex chemotherapy group (12.6% ± 9.1%) were significantly smaller than in the normal cortex chemotherapy group (60.7% ± 13.4%) (P=.0001).

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significantly fewer secondary follicles in the adult chemotherapy group than the adult control group (P=.009).

Apoptosis

Concerning apoptosis, a follicle was considered positive if the oocyte or >50% of GCs were stained for caspase-3 (18). In the control group, median (range) apoptosis rates were 27.5% (0–82.7) in prepubertal patients, 4.5% (0–60) in peripubertal patients, 0.0% (0–40) in postpubertal patients, and 13.1% (6.3–20.0) in adult patients. In the chemotherapy group, these figures were respectively 0.0% (0–55.6), 4.3% (0–21.8), 6.8% (0–42.9), and 4.8% (0–24.1). No significant difference was observed between controls and chemotherapy subjects in any age groups.

Activation

Follicle activation was investigated using the p-Akt marker. Activation rates per patient were based on the average percentage of stained area for all NGFs on each slide. In the control group, medians (range) were 0.3% (0.0–0.9) in prepubertal patients, 0.4% (0.1–2.5) in peripubertal patients, 1.0% (0.0–2.9) in postpubertal patients, and 0.7% (0.0–12.8) in adult patients. In the chemotherapy group, these medians (range) were respectively 2.0% (0.1–16.2), 0.9% (0.1–18.5), 6.1% (0.1–24.9), and 9% (0.1–20.4). In all age categories, numbers of activated follicles were systematically higher in the chemotherapy group than the control group, with this difference reaching statistical significance in the prepubertal group (P=.005).

Fibrosis

In the control group, means ± SDs of fibrotic area out of total cortex were 24.2% ± 9.8% in prepubertal patients, 21.3% ± 18.6% in peripubertal patients, 23.9% ± 14.6% in postpubertal patients, and 26.2% ± 0% in adult patients. In the chemotherapy group, they were respectively 39.6% ± 25.4%, 37.0% ± 22.6%, 33.1% ± 13.4%, and 30.7% ± 14.3%. Fibrotic areas

were systematically more substantial in the chemotherapy group than the control group in all age categories, but the difference was not significant (Fig. 3A).

Vascularization

In the control group, means ± SDs of vessel density were 72 ± 29 vessels/mm^2 in prepubertal patients, 63 ± 22 vessels/mm^2 in peripubertal patients, 76 ± 51 vessels/mm^2 in postpubertal patients, and 65 ± 72 vessels/mm^2 in adult patients. In the chemotherapy group, they were respectively 44 ± 24 vessels/mm^2, 43 ± 35 vessels/mm^2, 54 ± 21 vessels/mm^2, and 31 ± 21 vessels/mm^2. There was no statistical difference in total numbers of vessels between groups.

Immature vessel rates in the control group were 90.41% in prepubertal patients, 87.30% in peripubertal patients, 87.01% in postpubertal patients, and 95.35% in adult patients. In the chemotherapy group, they were respectively 88.64%, 86.05%, 90.74%, and 92.31%. No statistical difference was found in immature vessel rates or mature vessel rates between groups.

Atypical cortex group

Areas of atypical follicles (Fig. 1F) were observed in 4 out of 14 prepubertal patients in the chemotherapy group. In these 4 patients, the interval between the start of chemotherapy and OTC ranged from 1 day to 1 month. In these areas of atypical follicles, mean follicle density was 84,570 ± 8,837 follicles/mm^3. However, they all looked dead, but without any signs of apoptosis. The whole cortex of these patients was significantly less fibrotic (12.6% ± 9.1%) than cortex from other prepubertal chemotherapy patients (60.7% ± 13.4%) (P=.0001) (Fig. 3B).

DISCUSSION

One significant side effect of chemotherapy with gonadotoxic drugs like alkylating agents is iatrogenic POI, which impacts quality of life in women undergoing chemotherapy, reducing both reproductive capacity and systemic endocrine function (1–3, 6). Among fertility preservation options on offer, OTC followed by OTT can restore ovarian function in more than 95% of patients after reimplantation for a mean duration of 4–5 years, potentially stretching to 7 years depending on follicle density at OTC (1, 6) and yielding 30%–40% of live births (6, 22). In most cases, biopsies for OTC are taken before exposure to any chemotherapy, as recommended by the Edinburgh criteria, which exclude women aged over 15 years who have undergone chemotherapy (7). In some instances, however, fertility preservation cannot be performed before initiation of chemotherapy. This is the case in patients with lymphoma in whom surgery is contraindicated due to mass compression (1, 2), or in women with acute leukemia who run the risk of reintroduction of malignant cells into the ovarian bloodstream (23). In such cases, OTC may be performed between chemotherapy sessions or when the patient is in complete remission (leukemia) (22). As clearly stated in multiple studies (8, 23–26), it is indeed crucial that patients with leukemia be in complete remission at the time of OTC to avoid the presence of any malignant cells in their ovarian tissue. It also concerns patients who were initially treated with low-risk chemotherapy, but suffered a relapse or disease progression, requiring further chemotherapy or radiotherapy (like total body irradiation) at higher risk of gonadotoxicity (8, 27, 28). In patients who are between chemotherapy sessions, OTC should be considered over and above controlled ovarian stimulation and vitrification of oocytes, as we know that fertility outcomes are poor (29) and the risk of congenital anomalies cannot be excluded (30). To date, however, no congenital anomalies have been found in infants born after OTC and OTT, even when OTC was performed post-chemotherapy exposure, according to 2 large series (6, 31).

Recent studies (6, 8, 9) have demonstrated similar live birth rates ranging from 30% to 40% after OTT in individuals subjected to some chemotherapy before OTC. To understand why these initial chemotherapy sessions did not decrease pregnancy rates after OTT, we set out to evaluate the effects (tissular and histological) of nongonadotoxic doses of chemotherapy on ovarian tissue before OTC.

In our series, to avoid possible bias due to the heterogeneity of treatments administered in the chemotherapy group, only patients having recently (<3 months) been exposed to alkylating agents, known to be the most gonadotoxic drugs, were selected. Indeed, the North American Children's Oncology Group considers the risk of POI to be highest with alkylating agents at doses of >600 mg/m^2 busulfan, >60,000 mg/m^2 ifosfamide, and >7,500 mg/m^2 cyclophosphamide (14). In our study, it is noteworthy that doses administered before OTC were lower than the gonadotoxic limit. The highest ifosfamide dose administered was 60,000 mg/m^2 and the highest cyclophosphamide dose 3,400 mg/m^2.

According to the literature, chemotherapy affects the ovarian reserve through various pathways, as illustrated by multiple human and animal models (32, 33). The main 3 mechanisms are follicle apoptosis; overactivation of NGFs; and stromal damage (13). We investigated the effects of low doses of alkylating agents by studying these 3 mechanisms in ovarian tissue exposed to chemotherapy and comparing it with ovarian tissue free of chemotherapy. Moreover, to avoid any pathology-related bias, all our patients were age and pathology matched.

Histological features

In the literature, follicle density is lower in patients treated with alkylating agents (4, 32), with increased proportions of growing follicles and decreased NGF rates (33). In this study, however, patients exposed to chemotherapy exhibited neither a decline in follicle density, nor a significant change in follicle proportions. These results were similar to findings in a recent study by Labrune et al. (34), but contrary to another investigation where a significant downturn was observed (35). In the latter study, however, the number of patients was small (n = 5) and the mean age was high (29.8 years).

Secondary follicle proportions were lower in the chemotherapy group than the control group, and this difference

was significant in adult patients, suggesting that growing follicles could well be impaired by chemotherapy. In terms of their numbers, it is well established that growing follicles are more sensitive to chemotherapy (36), as their GCs are in the process of dividing, explaining the temporary amenorrhea patients experience after chemotherapy administration (13). As the interval between chemotherapy and OTC was less than 3 months in all cases, and the follicle cycle in human ovarian tissue is approximately 200 days (16), almost no secondary follicles were observed after chemotherapy exposure. Indeed, they were probably destroyed, explaining the difference observed with the group free of any chemotherapy. This reinforces the fact that OTC should be considered over ovarian stimulation when done soon after chemotherapy exposure. Nevertheless, the primordial follicle pool appears to be resistant to chemotherapy, and it is those follicles that will continue to grow after OTT.

Abnormal follicles exhibiting specific histological features, namely multinucleated oocytes and multi-oocyte follicles (Fig. 1A), were encountered in 89% of cases. Previous studies also witnessed high rates of abnormal follicles in prepubertal ovaries (15, 37, 38). These high rates were not influenced by chemotherapy in our study. Multi-oocyte follicles are probably a sign of abnormal folliculogenesis that disappears as the child matures. Indeed, a dramatic drop was observed in adult groups compared with prepubertal tissue.

Chemotherapy-induced damage: apoptosis

Most chemotherapeutic agents disrupt cell cycles and processes, destroying proliferating cells. They induce double-strand breaks in DNA, leading to either cell repair pathways and survival or, in case of impaired mechanisms, cell death by apoptosis (32, 39). This damage occurs in replicating cells, namely GCs in growing follicles, resulting in apoptosis with subsequent transient amenorrhea, as suggested by Kashi and Meirow (13). The role of apoptosis in NGFs is nevertheless contentious, with some studies showing double-strand breaks in oocytes exposed to cyclophosphamide along with apoptosis (40–43), and others failing to demonstrate any apoptotic markers in NGFs (11, 33, 44).

In our study, there was no difference in apoptosis rates (by caspase-3 staining) in either NGFs or growing follicles between patients exposed to chemotherapy and those not. This is in contrast with the recent literature, where high rates of apoptosis (assessed by TdT-mediated dUTP-biotin nick-end labeling) were reported by Devos et al. (36) in ovarian tissue exposed to chemotherapy before OTC. It should, however, be noted that this was observed after 24 hours of culture, which may itself influence survival of follicles. The absence of apoptosis in our study could be due to the timing between chemotherapy exposure and ovarian tissue retrieval, as apoptosis is a very fast-moving phenomenon. Indeed, proteins like caspases are expressed only fleetingly and the interval from initiation to completion of apoptosis can occur in as little as 2–3 hours (45). In our series, the shortest duration between chemotherapy administration and ovarian tissue harvesting was 24 hours, which could possibly fall outside the timeframe allowing us to track it.

Chemotherapy-induced damage: activation

Primordial follicle activation is regulated by 2 major factors: the phosphatidylinositol 3-kinase pathway leading to phosphorylation of mammalian target of rapamycin, which is intrinsic to the oocyte; and paracrine factors like anti-Müllerian hormone secreted by growing follicles, which are extrinsic (13). In our study, an increase in activation of NGFs was encountered in the chemotherapy group, which was significant in the prepubertal group. Meirow's team suggests that chemotherapy causes upregulation of the phosphatidylinositol 3-kinase pathway, thereby triggering activation of the NGF pool, which in turn induces burn-out of the follicle reserve (11, 13, 46, 47). This follicle activation was demonstrated in vivo in ovaries from recently treated (4–12 days of alkylating agents) patients (33), and in vitro in human ovarian cortex treated with cyclophosphamide (48). In our study, while higher rates of follicle activation were observed in the chemotherapy-treated group, it did not lead to a decrease in follicle density, even when chemotherapy was administered 3 months before biopsy. However, repeated treatments with cyclophosphamide may induce over-recruitment and incur more damage than single-dose treatments. Indeed, each dose triggers a new burst of activation and growth of follicles, which then become atretic or apoptotic as a consequence of subsequent treatment, causing ovarian reserve depletion (13). Importantly, it was shown that freeze/thawing of ovarian tissue does not alter apoptosis or activation rates (49), so outcomes observed at this stage of the study, up to 3 months after chemotherapy administration, are not affected or worsened after thawing.

Chemotherapy-induced damage: stromal damage (fibrosis)

In a comparison of chemotherapy-exposed patients and control subjects, we did not detect any significant difference in fibrosis rates. In 2007, Meirow described areas of focal follicle loss and fibrosis due to chemotherapy. His study hypothesized that chemotherapy causes blood vessel obstruction and, since the ovary has an end-artery vascularization system, induces ischemia, follicle loss, and subsequent fibrosis of the region (12). The apparent discrepancy between our series and that of Meirow could be the result of the same process at 2 different timepoints. In our series, a relatively short interval after chemotherapy exposure showed signs of ischemia but no fibrosis, whereas in Meirow's series, a later timepoint revealed a fibrotic cortex and loss of follicles. Devos et al. (36) also encountered no fibrosis after a short interval, reinforcing the notion that fibrosis takes 4–6 months after exposure to set in.

Limitations

The findings are reassuring, even if sample size, although the largest in the literature so far, is still limited. Although not significant, follicle density in our control subjects was lower

than in the chemotherapy groups. We do not have a clear explanation for this and believe that this issue may be addressed in further studies. Data on thawed tissue and in vivo results should be fully investigated to characterize ovarian tissue once reimplanted if chemotherapy was administered before OTC.

It would naturally be interesting to analyze ovarian tissue in the >3-month post-exposure window, which could provide further evidence on the lack of impact of lower doses of alkylating agents on the resting pool of primordial follicles. There are samples from patients who underwent OTC more than 3 months after chemotherapy, but the series in our database is too small to reach statistical significance, which is why these patients were not included in our study.

CONCLUSION

Our study did not demonstrate any major impact of low-dose chemotherapy on ovarian tissue, suggesting that cryopreserved ovarian tissue exposed to some chemotherapy is similar to ovarian tissue free of any chemotherapy. This corroborates clinical findings, as pregnancy rates after OTT were comparable in women with and without chemotherapy prior to OTC. Of course, the effects of chemotherapy on ovarian function are dose dependent and type dependent, and high doses of alkylating agents clearly have a deleterious effect on the ovarian reserve. In our study, all participants in the chemotherapy-exposed group were given alkylating agents at relatively low doses, leading us to conclude that some prior chemotherapy should no longer be considered a contraindication to OTC. OTC and OTT could therefore be proposed as the only available fertility preservation strategy so far to all patients in remission from leukemia or between treatments for other pathologies, even when some chemotherapy has already been administered.

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CRediT Authorship Contribution Statement

Lara Houeis: Conceptualization, data analysis, investigation, and writing of the original draft; **Graziella van der Plancke:** Data analysis; **Catherine Poirot:** Conceptualization, review and editing; **Luciana Cacciottola:** Review and editing; **Alessandra Camboni:** Resources; **Isabelle Brocheriou:** Resources; **Jacques Donnez:** Review and editing; **Marie-Madeleine Dolmans:** Conceptualization, supervision, project administration, review and editing.

Declaration of Interests

L.H. has nothing to disclose. G.v.d.P. has nothing to disclose. C.P. has nothing to disclose. L.C. has nothing to disclose. A.C. has nothing to disclose. I.B. has nothing to disclose. J.D. is a member of the scientific advisory board of Theramex outside the submitted work, and has nothing to disclose in relation to the article. M.-M.D. has nothing to disclose.

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