FULL LENGTH ARTICLE

Chemotherapy-induced diminished murine ovarian reserve model and impact of low-dose chemotherapy on fertility

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Objective: To establish a murine model of chemotherapy-induced diminished ovarian reserve (DOR) and investigate residual fertility after chemotherapy exposure.
Design: Two different chemotherapy protocols were tested to establish a valid DOR model by comparing follicle densities in mice given either protocol or physiological solution. An ovarian stimulation protocol was then selected from among different gonadotropins by counting the number of day 2 embryos obtained from normal mice. Finally, DOR mice were stimulated 5 and 8 weeks after chemotherapy with the chosen gonadotropin protocols, and day 2 embryos were recovered after mating, as was ovarian tissue for further immunohistologic analyses.
Subjects: Seventy-two Naval Medical Research Institute mice.
Exposure: Two different chemotherapy protocols.
Main Outcome Measures: This study compared day 2 embryo counts in both normal and chemotherapy-induced DOR mice. Ovarian histology and morphology were also investigated by follicle counting and classification, as was immunostaining for apoptosis (cleaved caspase-3), activation (phospho-Akt), and proliferation (Ki67).
Results: A dose of 12 mg/kg of busulfan (Bu) + 120 mg/kg of cyclophosphamide (Cy) was chosen to establish the DOR model as it significantly reduced the ovarian reserve compared to both control mice (physiological solution) and the 1.2 mg/kg of Bu + 12 mg/kg of Cy protocol, without depleting it completely. When stimulated with 3.75 IU of Menopur, normal mice produced significantly more embryos than DOR mice given 12 mg/kg of Bu + 120 mg/kg of Cy (41.40 ± 14.74 vs. 23.67 ± 15.55 day 2 embryos). Although the follicle count was statistically diminished after single-dose chemotherapy administration, the remaining follicles did not display any difference in terms of apoptosis, activation, or proliferation rates.
Conclusion: We successfully established a chemotherapy-induced DOR model using 12 mg/kg of Bu + 120 mg/kg of Cy, as evidenced by lower, but not completely depleted, follicle numbers and fewer retrieved embryos. Histologic study of ovarian tissue exposed to DOR-inducing chemotherapy revealed that surviving follicles were of the similar quality as tissue not exposed to chemotherapy. (F S Sci® 2025;■:■–■. ©2025 by American Society for Reproductive Medicine.)

Key Words: Ovarian tissue, chemotherapy, alkylating agents, diminished ovarian reserve, fertility

F&S SCIENCE CLINICAL QUICK TAKE

What clinical problem is addressed by these studies?

- While recent advances in cancer treatment have significantly improved patient survival rates, one of their major side effects is development of ovarian dysfunction, ranging from a diminished ovarian reserve (DOR) to complete premature ovarian insufficiency. Many murine models of premature ovarian insufficiency exist; however, there are only very few of DOR.

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What are the key findings?

- Our proposed model of DOR proved effective using 12 mg/kg of busulfan + 120 mg/kg of cyclophosphamide to induce a diminished, but not depleted, ovarian reserve, as evidenced by histology and embryo count after ovarian stimulation.

How do these findings apply to human fertility or the reproductive process?

- Establishment of a chemotherapy-induced DOR model will allow further studies into this serious issue affecting women and into development of novel gonadoprotective approaches.

Recent advances in cancer treatment have significantly improved patient survival rates, representing a major achievement in oncology. However, these therapeutic advances are not without their short- and long-term side effects (1). One of the most concerning issues is the potential impact on reproductive and endocrine function, particularly development of ovarian dysfunction (2).

Many patients offered ovarian tissue cryopreservation before chemotherapy exposure will present with premature ovarian insufficiency (POI) or premature ovarian failure; however, a significant proportion will only present with a diminished ovarian reserve (DOR), this number increasing further with time after chemotherapy (3). The most severe form of ovarian dysfunction is defined by postmenopausal levels (>25 IU/L) of follicle-stimulating hormone and/or secondary amenorrhea lasting ≥4 months in women aged <40 years (4, 5). While there is a semantic distinction between "failure" and "insufficiency," there is a consensus on using them interchangeably as synonyms, with a preference toward the term POI. At the milder end of the spectrum is DOR, where patients retain endocrine function and some fertility (6).

The effects of chemotherapy on female reproduction are highly detrimental as they can lead to infertility, hormonal imbalances, and other complications. They have been documented since the 1970s in human and murine tissue (7–9). The most highly gonadotoxic drugs are alkylating agents, including cyclophosphamide (Cy), the most notorious of all (1, 8, 10, 11). Mechanisms underlying ovarian damage due to chemotherapeutic drugs are well established and multifaceted (12). A number of studies have evaluated the impact of Cy on human ovarian tissue (12), showing that it induces direct loss of follicles through apoptosis (13) and indirect loss through overactivation and premature growth of primordial follicles (14, 15). This leads to depletion of the ovarian reserve and, ultimately, exhaustion of both endocrine function and reproductive function depending on dose and patient age (2, 7, 16, 17). In addition to quantitative and qualitative alterations to the follicle pool, alkylating agents cause vascular damage and subsequent fibrosis within ovarian tissue (18, 19). In murine studies, the direct effects of Cy exposure are well documented and known to induce apoptosis in growing follicles, as well as vascular damage (20). It is, however, unclear whether there is also indirect loss through overactivation (12).

There are many murine models of chemotherapy-induced POI in the literature (21) but very few chemotherapy-induced DOR models (22, 23). Chemotherapy-triggered follicle loss is dose-dependent, and not all patients undergoing chemotherapy develop POI. Some may exhibit only DOR and retain some residual ovarian function, meaning that they may not always need to rely on previously retrieved oocytes or ovarian tissue for fertility preservation. Efforts to establish DOR models have focused on using lower doses of alkylating agents than those associated with POI induction, although discrepancies remain among the existing models.

Establishing a chemotherapy-induced DOR model will, therefore, pave the way to fresh insights into this medical condition and help develop gonadoprotective approaches. The goal of our study was to generate a model for DOR induction considering dose and timing of drug administration and investigate residual murine fertility potential in terms of ovarian reserve and embryo rates.

MATERIALS AND METHODS

Experimental design and aim of our study

Our study was divided into three experimental phases (Fig. 1).

- Phase 1: identifying the chemotherapy dose generating a significant reduction in the ovarian reserve
- Phase 2: investigating ovarian stimulation protocols to determine which produces the best embryo yield in normal mice
- Phase 3: exploring whether chemotherapy-induced DOR affects embryo yield after ovarian stimulation

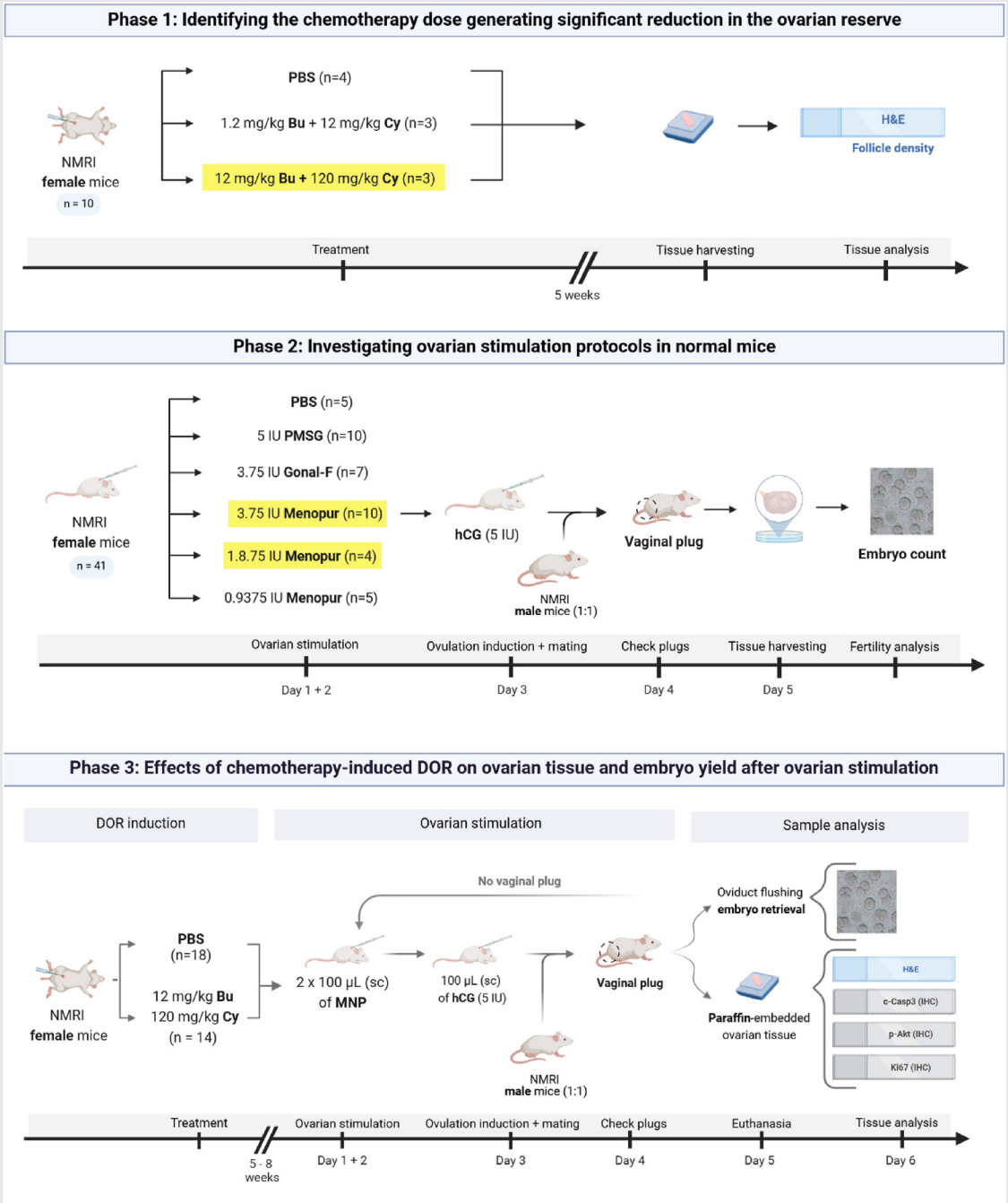
Mouse origin

Seventy-two 8-week-old female Naval Medical Research Institute mice (Charles River Laboratories, Saint Germain Nuelles, France) were used for the study. Appropriate housing and breeding conditions were applied, as previously reported (21). Animal welfare was fully respected and complied with all guidelines endorsed by the Committee on Animal Research of the Université Catholique de Louvain (reference 2023/UCL/MD/01).

Phase 1: identifying the chemotherapy dose generating a significant reduction in the ovarian reserve

Diminished ovarian reserve was induced by administering different doses of chemotherapy. In brief, 8-week-old female Naval Medical Research Institute mice were treated with physiological solution (n = 4) or a chemotherapy regimen involving either a single intraperitoneal injection of 1.2 mg/kg of busulfan (Bu) and 12 mg/kg of Cy (n = 3) (lower-dose chemotherapy group) or 12 mg/kg of Bu and 120 mg/kg of Cy (n = 3) (higher-dose chemotherapy group), resuspended in dimethyl sulfoxide and diluted in phosphate-buffered saline to reach a 100-μL volume to inject. Murine ovaries were then retrieved 5 weeks after chemotherapy administration. They were fixed in 4% neutral-buffered formaldehyde,

FIGURE 1



Phase 1: Identifying the chemotherapy dose generating a significant reduction in the ovarian reserve. Ten mice underwent either a single intraperitoneal (IP) injection of physiological solution- (n = 4), a single IP injection of 1.2 mg/kg of busulfan (Bu) + 12 mg/kg of cyclophosphamide (Cy) (n = 3, lower-dose chemotherapy group), or a single IP injection of 12 mg/kg of Bu + 120 mg/kg of Cy (n = 3, higher-dose chemotherapy group). Five weeks later, the ovaries were retrieved and follicle densities were assessed. The higher-dose chemotherapy protocol was selected as it effectively induced a diminished ovarian reserve (DOR) (highlighted in yellow). Phase 2: Investigating ovarian stimulation protocols in normal mice. Forty-one normal mice were subjected to various protocols: 5 were given physiological water as controls and the others received 5 IU of pregnant mare serum gonadotropin (PMSG) (n = 10), 3.75 IU of Gonal-F (n = 7), 3.75 IU of Menopur (MNP) (n = 10), 1.875 IU of MNP (n = 4), or 0.9375 IU of MNP (n = 5) for ovarian hyperstimulation. Protocols using 3.75 IU of MNP and 1.875 IU of MNP were selected (highlighted in yellow). Phase 3: Effects of chemotherapy-induced DOR on ovarian tissue and embryo yield after ovarian stimulation. We compared ovarian tissue and embryo yield after stimulation using either 3.75 IU of MNP, 1.875 IU of MNP, or physiological solution between DOR mice (according to the selected protocol in phase 1) and normal mice. Created with BioRender. H & E = hematoxylin and eosin; hCG = human chorionic gonadotropin; IHC = immunohistochemistry; NMRI = Naval Medical Research Institute; p-Akt = phospho-Akt; PBS = phosphate-buffered saline.

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embedded in paraffin, and cut into 5-μm sections, which were stained with hematoxylin and eosin for follicle density assessment.

Phase 2: investigating ovarian stimulation protocols to determine which produces the best embryo yield in normal mice

Forty-one normal mice were subjected to various protocols: 5 were given physiological water as controls, and the others received 5 IU of pregnant mare serum gonadotropin (PMSG) (n = 10), 3.75 IU of Gonal-F (n = 7), 3.75 IU of Menopur (n = 10), 1.875 IU of Menopur (n = 4), or 0.9375 IU of Menopur (n = 5) for ovarian stimulation. The total gonadotropin dose was split in half and administered subcutaneously for 2 days. For instance, mice receiving 5 IU of PMSG were given injections of 2.5 IU of PMSG on both days 1 and 2. Forty-eight hours later, they were subcutaneously injected with 5 IU human chorionic gonadotropin (brand name, Ovitrelle), followed by mating with fertile males. Female mice displaying a vaginal plug within 16–20 hours of mating were euthanized 48 hours after human chorionic gonadotropin injection to recover their ovaries and collect embryos from the oviduct.

The animals were euthanized by cervical dislocation. Bilateral ovaries and oviducts were recovered and separated by cutting. The ovaries were weighed and measured for size and then fixed in 4% neutral-buffered formaldehyde for analysis. The oviducts were placed in M2 medium in a petri dish, and then, embryos from the oviducts were expelled by inserting a 34-gauge blunt microneedle into the infundibulum and flushing medium through the oviduct. Embryos were identified and classified under a microscope into 2-cell embryos or degenerated embryos.

Phase 3: exploring whether chemotherapy-induced DOR affects embryo yield after ovarian stimulation

After DOR induction according to the selected protocol (12 mg/kg of Bu + 120 mg/kg of Cy), DOR mice underwent ovarian stimulation also in line with the chosen protocol. Five weeks after chemotherapy, they were stimulated using 3.75 IU of Menopur (n = 16), 1.875 IU of Menopur (n = 3), or physiological solution (n = 2) and mated with males with proven fertility. If no vaginal plug was found after stimulation, ovulation, and mating, they were restimulated with 3.75 IU of Menopur 2 weeks later, namely, 8 weeks after chemotherapy administration. Embryo counting was performed as explained earlier, as were ovarian retrieval and analysis. A timeline of the experiment is shown in Figure 1.

Outcome measurement

We evaluated the number of mice with vaginal plugs and recorded the embryo count and ovarian weight and size. Ovarian tissue from all mice was examined for follicle count, and follicles were classified by histology. Follicle proliferation was assessed using Ki67, apoptosis through caspase-3, and activation via phospho-Akt (p-Akt) immunohistochemical staining.

Ovarian tissue processing

Histology. After fixation in 4% formaldehyde for 24 hours, the ovarian tissue was embedded in paraffin and serially cut into 5-μm–thick sections. Every fifth section was stained with hematoxylin and eosin (Sigma-Aldrich, Machelen, Belgium) to establish follicle count, density, and classification. For histologic evaluation, stained slides were scanned with the Pannoramic P250 digital slide scanner (3DHISTECH, Budapest, Hungary) and analyzed using CaseViewer v2.4 (3DHISTECH). Follicle count was determined by every fifth section from one end of the ovary to the other. Only follicles with a visible oocyte nucleus were counted, ensuring that no preantral follicles were counted twice. They were then classified into primordial, intermediate, primary, secondary, antral, or atretic (defined as pyknosis, granulosa cell layer disorganization, and/or oocyte fragmentation) (22–25).

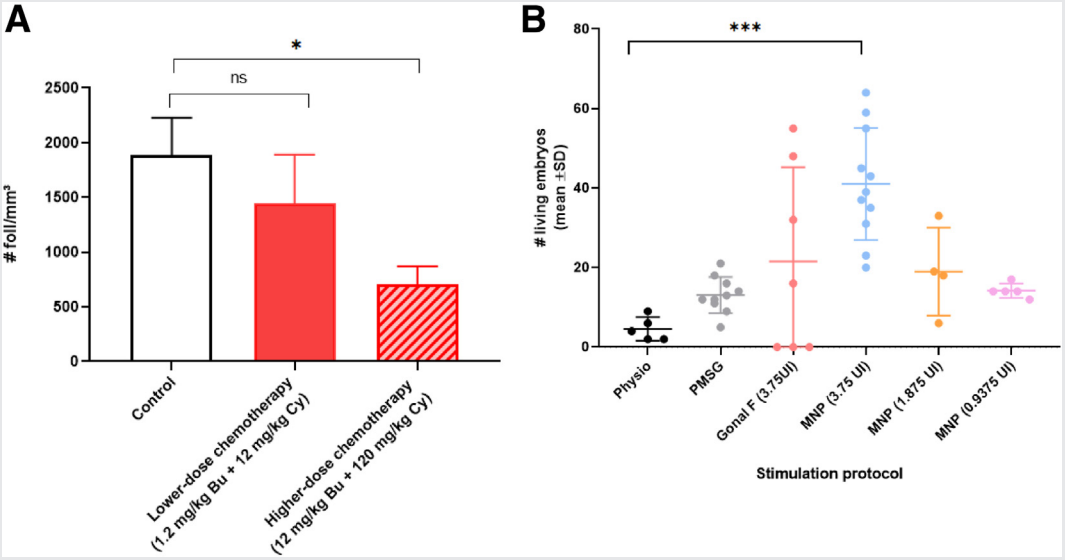
Immunohistochemistry. Protocols for immunohistochemical staining have been previously described in detail (26). In brief, sections were deparaffinized with Histosafe (Yvsolab SA, Beerse, Belgium) and rehydrated in hydrogen peroxide (Merck, Darmstadt, Germany) for 30 minutes at room temperature. After demasking in citrate buffer (pH of 6) for 75 minutes at 98 °C, nonspecific binding sites were blocked by incubation with normal goat serum for 30 minutes at room temperature. The sections were then incubated overnight at 4 °C with primary antibodies: mouse monoclonal anti-mouse (anti-human) Ki67 antibody (M7240, 1:100 dilution; Dako, Carpinteria, CA); rabbit polyclonal anti-mouse (anti-human) cleaved caspase-3 antibody (9661S, 1:100 dilution; Cell Signaling Technology, Danvers, MA); or rabbit monoclonal anti-p-Akt antibody (4060P, 1:100 dilution; Cell Signaling Technology). The slides were subsequently incubated for 60 minutes at room temperature with goat anti-rabbit secondary antibody (Envision anti-rabbit system HRP [horseradish peroxidase]; Dako). Diaminobenzidine (Dako) was used as a chromogen, and hematoxylin was used as a counterstain. Negative controls were incubated with universal negative controls (IS600; Dako).

Two nonconsecutive sections at least 200 μm apart were examined per sample. Stained slides were then scanned with the Pannoramic P250 digital slide scanner (3DHISTECH) and analyzed using CaseViewer v2.4 (3DHISTECH). Only nongrowing follicles (NGFs) (primordial and intermediate follicles) were assessed for Ki67. Follicles containing at least one granulosa cell staining positive for Ki67 were classified as proliferative (27). For caspase-3 labeling, follicles at all stages were evaluated and classified as apoptotic when >50% of granulosa cells and/or the oocyte stained positive for caspase-3 (28). Sections labeled with p-Akt were investigated using Fiji software. After delineation and annotation of NGFs, the rates of follicle activation were quantified by calculating the positive-stained area divided by total area of NGFs (26) with computer assistance.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software, v8 (GraphPad Software, Inc., La Jolla, CA). The Student's

FIGURE 2



(**A**) Diminished ovarian reserve (DOR) model establishment: lower-dose chemotherapy vs. higher-dose chemotherapy. Higher-dose chemotherapy mice (n = 3) showed a significantly decreased follicle density compared with the control group (n = 4) and the lower-dose chemotherapy group (n = 3). The higher-dose model was chosen for DOR establishment in the subsequent experiment. (**B**) Ovarian stimulation protocol establishment: the 3.75 IU of Menopur (MNP) protocol was the only one yielding significantly more embryos than the control group; thus, this gonadotropin was selected to stimulate DOR mice. Bu = busulfan; Cy = cyclophosphamide; PMSG = pregnant mare serum gonadotropin.

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unpaired *t*-test was applied to compare follicle density, follicle classification, and expression of different immunohistochemical markers between groups. In case of abnormal distribution or small sample size, the Mann-Whitney *U* test was used. $P<.05$ was considered statistically significant.

RESULTS

Phase 1: identifying the chemotherapy dose generating a significant reduction in the ovarian reserve

In the control group, the mean follicle density was 1,884 ± 343 follicles/mm^3. In the lower-dose chemotherapy group (1.2 mg/kg of Bu + 12 mg/kg of Cy), the mean follicle density was 1,444 ± 446 follicles/mm^3 and showed no statistically significant difference with the control group.

In the higher-dose chemotherapy group (12 mg/kg of Bu +120 mg/kg of Cy), the mean follicle density was 704 ± 163 follicles/mm^3, which was significantly ($P=.0125$) lower than in the control group. This second treatment protocol was, therefore, selected and used to investigate residual fertility (Fig. 2A).

Phase 2: investigating ovarian stimulation protocols to determine which produces the best embryo yield in normal mice

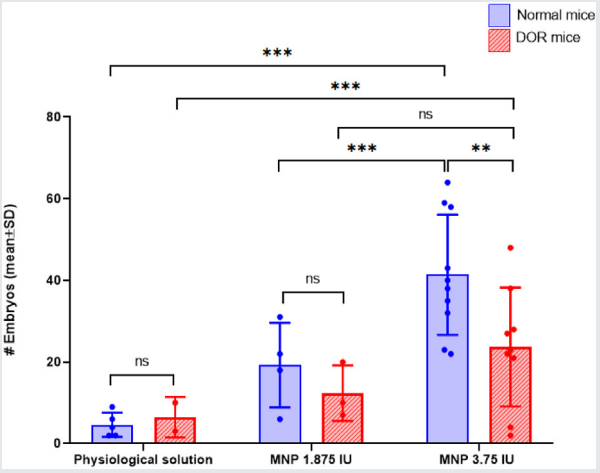
Normal mice were treated with different protocols of gonadotropins. The day 2 embryo count for each stimulation protocol was the following: 4.6 ± 2.9 embryos when stimulated with physiological solution (controls); 13 ± 4.5 embryos when stimulated with PMSG; 21.57 ± 23.64 embryos when stimulated with Gonal-F; 41 ± 14.11 embryos when stimulated with 3.75 IU of Menopur; 19 ± 11 embryos when stimulated with 1.875 IU of Menopur; and 14 ± 1.79 when stimulated with 0.9375 IU of Menopur.

There was a significant ($P<.0001$) increase in the number of day 2 embryos retrieved when using 3.75 IU of Menopur compared with that in the control group (Fig. 2B). For the sake of homogeneity, it was decided to perform all further stimulations with 3.75 and 1.875 IU of Menopur only.

Phase 3: exploring whether chemotherapy-induced DOR affects embryo yield after ovarian stimulation

Number of embryos. The number of embryos retrieved from the animals by flushing was used to evaluate residual fertility potential (Fig. 3). When stimulated with physiological solution, the means ± SDs of embryos were 4.6 ± 2.96 in the control group and 6.5 ± 4.95 in the chemotherapy-treated group. The difference between the two groups was not significant. When stimulated with 1.875 IU of Menopur, the means ± SDs of embryos were 19.25 ± 10.37 in the control group and 12.33 ± 6.81 in the chemotherapy-treated group. Again, the difference between groups was not significant. However, when stimulated with 3.75 IU of Menopur, the means ± SDs of embryos were 41.40 ± 14.74 in the control group and

FIGURE 3



Numbers of embryos collected after ovarian stimulation with physiological solution, 1.875 IU of Menopur (MNP), or 3.75 IU of MNP in diminished ovarian reserve (DOR) mice compared with normal mice. Diminished ovarian reserve mice produced significantly fewer embryos than normal mice when stimulated with 3.75 IU of MNP. In normal mice, the increase in embryo numbers was significant upon stimulation with 3.75 IU of MNP compared to 1.875 IU of MNP. In DOR mice, there was no difference in numbers of embryos between stimulation protocols.

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23.67 ± 15.55 in the chemotherapy-treated group. The difference between these two groups was significant (P=.0127), exhibiting a decline in fertility when exposed to chemotherapy. Moreover, normal mice showed a statistically significant increase when stimulated with 3.75 IU of Menopur compared to 1.875 IU of Menopur (P=.005), whereas DOR mice did not show any statistical increase when stimulated at the higher dose.

Follicle count. The mean follicle counts per ovary were 820 ± 355 in the control group, 347 ± 100 in the 5-week chemotherapy group, and 299 ± 149 in the 8-week chemotherapy group. There was a significant difference between controls and both the 5-week (P=.0009) and 8-week (P<.0001) chemotherapy groups but no difference between 5 and 8 weeks of treatment (Fig. 4A).

Follicle morphology

Viable follicles. We investigated the proportion of viable follicles out of all follicles. The means ± SDs of viable follicles (Fig. 4B) were 58.55 ± 16.11% in the control group, 68.98 ± 19.83% in the 5-week chemotherapy group, and 51.70 ± 17.58% in the 8-week chemotherapy group. There was no significant difference in follicle viability according to chemotherapy exposure or duration.

Nongrowing follicles. The NGF proportions were calculated out of the population of viable follicles. The means ± SDs of NGFs (Fig. 4C) were 60.14 ± 11.80% in the control group, 55.99 ± 13.40% in the 5-week chemotherapy group, and 54.16 ± 22.00% in the 8-week chemotherapy group. No significant difference in NGFs was observed between mice exposed to chemotherapy and those not.

Growing follicles: primary, secondary, and antral. The proportions of primary, secondary, and antral follicles were also calculated out of the whole population of viable follicles (Fig. 4C). The means ± SDs of primary follicles were 33.98 ± 9.32% in the control group, 20.18 ± 8.78% 5 weeks after chemotherapy, and 20.72 ± 12.21% 8 weeks after chemotherapy. There were significantly (P=.0366) fewer primary follicles in the 8-week chemotherapy group than in the control group. Secondary follicles showed means ± SDs of 23.52 ± 11.02% in the control group, 17.94 ± 11.37% in the 5-week chemotherapy group, and 19.56 ± 15.28% in the 8-week chemotherapy group. Finally, the means ± SDs of antral follicles were 3.871 ± 3.191% in controls, 5.887 ± 4.368% after 5 weeks, and 5.552 ± 5.301% after 8 weeks. As shown in Figure 4C, there was a decrease in the number of growing follicles after chemotherapy, albeit not significant, regardless of timing.

Apoptosis. A follicle was considered positive for apoptosis if the oocyte or >50% of granulosa cells were stained for caspase-3 (28). The means ± SDs of the apoptosis rate (Fig. 4D) were 40.74 ± 16.16% in the control group, 31.02 ± 19.83% in the 5-week chemotherapy group, and 48.30 ± 17.58% in the 8-week chemotherapy group. No significant difference was observed between controls and chemotherapy-treated mice.

Activation. Follicle activation was assessed using the p-Akt marker. The activation rates per mouse were based on the average percentage of stained area for all NGFs on each slide. The means ± SDs of the activation rate (Fig. 4E) were 6.085 ± 4.728% in the control group, 3.199 ± 5.105% 5 weeks after chemotherapy, and 6.746 ± 2.794% 8 weeks after chemotherapy. There was no significant difference between the control and treatment groups.

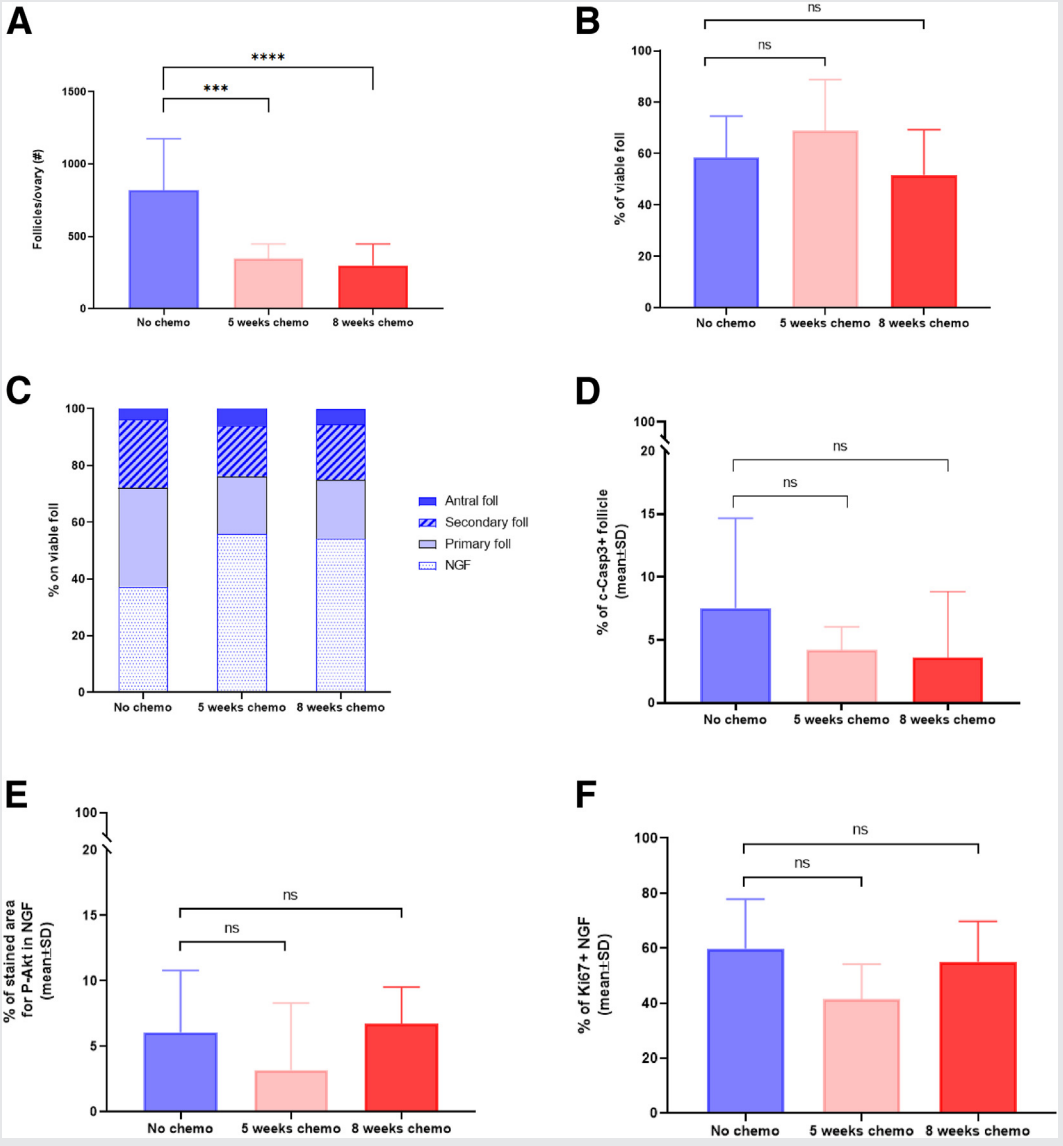
Proliferation. Follicle proliferation was determined by considering NGFs as proliferating if they contained at least one positive Ki67-labeled granulosa cell. Proliferation rates per mouse were calculated as the ratio of positive-stained NGFs to the total number of NGFs. The means ± SDs of the proliferation rate (Fig. 4F) were 59.88 ± 17.98% in the control group, 41.58 ± 12.61% in the 5-week chemotherapy group, and 55.09 ± 14.77% in the 8-week chemotherapy group. No significant difference in the proliferation rates was observed between exposed and nonexposed animals.

DISCUSSION

Establishment of an experimental chemotherapy-induced DOR model

The effects of chemotherapy, particularly Cy, on fertility and ovarian function have been observed in human tissue and multiple murine studies (8, 20, 29, 30). Indeed, there are many murine models of both premature ovarian failure and POI but very few models of DOR to be found in the literature.

FIGURE 4



(**A**) Follicle count: there was a significant decrease in follicle count at 5 and 8 weeks after chemotherapy administration at a dose of 12 mg/kg of busulfan + 120 mg/kg of cyclophosphamide compared to the control group. (**B**) Viable follicle rates: no difference was observed between mice exposed to 5 weeks of chemotherapy, those exposed to 8 weeks of chemotherapy, and the control group. (**C**) Follicle classification: no significant difference was observed in nongrowing follicles (NGFs); however, there was a decrease in the number of growing follicles after chemotherapy, albeit not significant, regardless of timing. (**D**) Apoptosis rates: no difference was found between mice exposed to 5 weeks of chemotherapy, those exposed to 8 weeks of chemotherapy, and the control group. (**E**) Activation rates: no difference was noted between mice exposed to 5 weeks of chemotherapy, those exposed to 8 weeks of chemotherapy, and the control group. (**F**) Proliferation rates: no difference was observed between mice exposed to 5 weeks of chemotherapy, those exposed to 8 weeks of chemotherapy, and the control group. p-Akt = phospho-Akt.

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As chemotherapy-induced follicle loss is dose-dependent, studies have aimed to develop the DOR model by administering lower doses of alkylating agents than those used to induce POI.

In a study by Buigues et al. (31), immunodeficient mice were injected with either a high dose of alkylating agents (12 mg/kg of Bu + 120 mg/kg of Cy) or a low dose of alkylating agents (1.2 mg/kg of Bu + 12 mg/kg of Cy). The high-dose protocol induced an 87% decrease in the follicle count, which was considered as POI induction, whereas the low-dose treatment triggered a 33% reduction, considered as DOR.

In a study by Salvatore et al. (32), CD-1 mice were given higher doses than those by Buigues (31). The high dose (30 mg/kg of Bu + 120 mg/kg of Cy) induced a 90% decrease,

considered as POI, whereas the low dose (12 mg/kg of Bu + 120 mg/kg of Cy) led to a 75% decrease, considered as DOR (32). Interestingly, the "low dose" in the study by Salvatore et al. (32) corresponds to the "high dose" in the study by Buigues et al. (31), and indeed, the definitions of POI and DOR vary in terms of follicle count according to the two articles. Although some models have been established, discrepancies remain. In our study, a dose of 1.2 mg/kg of Bu + 12 mg/kg of Cy did not really affect the ovarian reserve. However, 12 mg/kg of Bu + 120 mg/kg of Cy was enough to significantly reduce it but not deplete it. This was evidenced by the fact that embryos could still be obtained, despite their reduced numbers. It should be noted that different mouse strains were used in the different models.

The model we propose appears to be highly effective for DOR. Indeed, in our study, mice exposed to chemotherapy at a dose of 12 mg/kg of Bu + 120 mg/kg of Cy exhibited lower follicle density than did control mice. Nevertheless, they were able to form embryos, albeit at a lower rate than their control counterparts. We can conclude that their ovarian reserve was diminished but not completely depleted and that our model is a valid approach to study DOR, having encountered remaining normal follicles and residual fertility.

Evaluation of fertility and the ovarian reserve

Our data showed that DOR mice are able to produce fertilizable oocytes when hyperstimulated.

Relationship between the ovarian reserve and fertility?

Interestingly, the number of embryos retrieved from DOR mice on stimulation with low-dose gonadotropins (1.875 IU of Menopur) or upon mating with no stimulation was similar to that in the control group. This shows that DOR does not necessarily correlate with lower fertility rates nor a poor ovarian response to gonadotropins. It was only when stimulated with high-dose gonadotropins (3.75 IU of Menopur) that the difference between DOR and normal mice emerged, with significantly fewer embryos in the DOR group than in the control group. This suggests that, as opposed to normal mice, DOR mice are unable to recruit more follicles when stimulated at high doses, as their ovarian reserve is reduced, making them poor responders at these doses. This lends further weight to our DOR model as it may mimic clinical data, where patients with a low antral follicle count do not benefit from high-dose gonadotropins for in vitro fertilization (IVF) (33).

To summarize, patients with DOR may be poor responders when undergoing ovarian stimulation due to their lower ovarian reserve. Indeed, according to the POSEIDON criteria (34, 35), poor responders to IVF can be divided into four groups. Groups 1 and 2 are both characterized by a normal ovarian reserve; however, fewer oocytes than expected are retrieved after stimulation. They are, therefore, "unexpected" poor responders, with possible resistance to follicle-stimulating hormone. On the other hand, groups 3 and 4 show the typical features of DOR (low antimüllerian hormone level and antral follicle count), and our model mimics these subsets where higher gonadotropin doses do not yield benefits. Indeed, these subjects recruit fewer follicles; hence, fewer oocytes are recovered (in some cases, none or the cycle canceled) because their ovarian reserve is too low, making them DOR-induced poor responders.

Histologic analyses

Our histologic analyses showed that the remaining follicles in DOR mice were similar in terms of viability, apoptosis rates, activation rates, and proliferation rates, suggesting that follicles surviving after chemotherapy are of similar quality as those not exposed to chemotherapy, despite their lower numbers. In terms of follicle classification, growing follicles were observed at the primary, secondary, and antral stages. As the full life span of follicles in this species is just 3 weeks (36) and our first analyses were conducted 5 weeks after chemotherapy, the growing follicles we observed were at the primordial stage at the time of chemotherapy and were, therefore, safe from its harmful effects.

Limitations

It is important to note that choosing the mating strategy as opposed to the IVF approach could be a point of concern as inconsistency due to male fertility factors or other variables may be introduced. However, only males with proven fertility were used in this study.

Importantly, we observed a decline in follicle count 8 weeks after chemotherapy. It should, nevertheless, be acknowledged that the ovaries analyzed after 8 weeks of chemotherapy were from different mice than those analyzed after 5 weeks. Indeed, we let them go on to 8 weeks as mating failed at 5 weeks. Mating proved successful in some mice at 8 weeks, meaning that they were not infertile.

CONCLUSION

In conclusion, the DOR model developed in this study proved to be effective at determining residual fertility in mice exposed to chemotherapy. This will pave the way for future investigations in terms of gonadoprotective approaches.

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CRediT Authorship Contribution Statement

Lara Houeis: Writing – original draft, Investigation, Data curation, Conceptualization. **Graziella van der Plancke:** Writing – original draft, Data curation. **Jen-Yu Wen:** Data curation. **Luciana Cacciottola:** Writing – review & editing, Conceptualization. **Jacques Donnez:** Writing – review & editing. **Marie-Madeleine Dolmans:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of Interests

L.H. has nothing to disclose. G.v.d.P. has nothing to disclose. J.-Y.W. has nothing to disclose. L.C. has nothing to disclose. J.D. has nothing to disclose. M.-M.D. has nothing to disclose.

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