Article

Exposure of Early Postnatal Oocytes to Chemotherapy Alters the Potential Ovarian Reserve, According to an Ex Vivo Mouse Model

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Abstract: Current safety data on chemotherapy during pregnancy are based on studies which focus on the mother and do not explore reproductive health and fecundity potential within the exposed offspring. We designed this randomized ex vivo animal study to evaluate the effect of chemotherapy on the developing ovarian reserve in the exposed offspring. Specimens (100 postnatal day zero C57BL/6 mouse ovaries) were randomized to control or chemotherapy drug exposure and maintained in a hanging well organ culture. Murine ovarian reserve establishment mirrors activity seen in the human fetus but with a significant time shift of the transition to meiotic arrest to the postnatal period. Exposures included: doxorubicin, cyclophosphamide, paclitaxel, docetaxel, and cisplatin. Doxorubicin resulted in a significant loss of 95.2% ($p < 0.0001$) of oocyte density compared to controls. Cyclophosphamide also caused depletion of 50.5% ($p < 0.0001$) of oocyte density. Cisplatin, docetaxel, and paclitaxel all demonstrated unique phenotypical changes on the ovaries and their oocytes, without a significant decrease in oocyte density over a five-day exposure. Exposure to chemotherapy may result in profound loss of oogonia during the transition to mature oocytes.

Keywords: ovarian reserve; oocytes; pregnancy; fetal; chemotherapy; cancer; doxorubicin; cyclophosphamide; paclitaxel; docetaxel; cisplatin

1. Introduction

US data on new cancer cases in 2021 estimate that 4.6% of all new cases will occur in young people aged 15 to 39 [1]. This reproductive age cohort carries a high risk of pregnancy diagnosed at the time, shortly before, or after the diagnosis of their malignancy. The most common types of cancer in this age group include breast, cervical, and hematologic cancers [1].

Fortunately with active treatment, no increased risk of cause-specific death has been seen in pregnant women diagnosed with cancer during pregnancy [2]. Furthermore, when advised to consider termination to expeditiously move forward with oncologic treatment less than 50% of women choose a therapeutic abortion [3,4]. Cancer associated with pregnancy has limited available epidemiologic data, but the impact of chemotherapy on the fetus seems to vary based on the agent, duration, and the gestational period of exposure [5]. Neoadjuvant chemotherapy followed by interval surgery is now acceptable if not preferable for ovarian, breast, and other advanced stage cancers [6].

Chemotherapy is not recommended during the first trimester due to a 20% risk of developing congenital malformations [3,4]. While most of the data to date suggest an

increased risk of preterm delivery (both iatrogenic and spontaneous) in children exposed to chemotherapy in utero during the second and third trimesters, these offspring do not appear to demonstrate a significant difference in congenital anomalies, cardiac function, or cognitive abilities [7–16]. However, ovarian reserve and number of oocytes has not been as well studied. As illustrated in Figure 1, the various organ systems have distinct time periods for organogenesis and maturation.
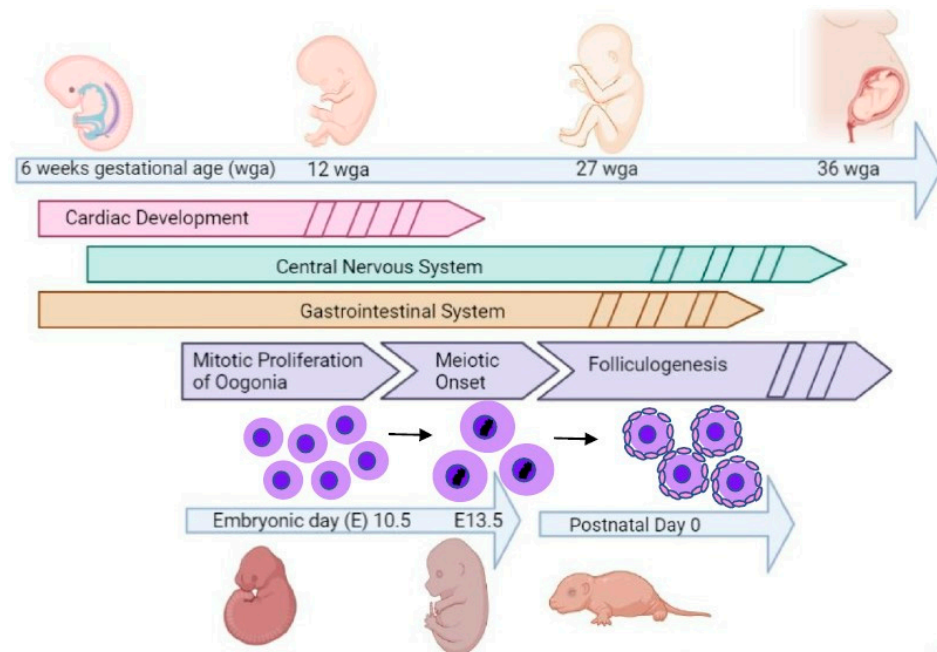

Figure 1. Oocyte Development in the human fetus versus the mouse.

Reproductive health with fertility and fecundity in offspring is unlikely to be represented on 10- or even 20-year follow-up of offspring of the treated maternal fetal couplet. The impact of an infertility diagnosis has been demonstrated in be correlated with increased anxiety and depressive symptomatology in women and their partners [17]. While medical treatment of early menopause with hormone replacement treatment may ameliorate vasomotor symptoms as deserve decrease in ovarian reserve occurs, treatments such as oocyte cryopreservation and ovarian tissue cryopreservation are much more effective when utilized while the individual is still have regular menses [18]. Discussion of and access to fertility preservation is recommended with a cancer diagnosis for adolescent and adult females from a medical, legal, ethical standpoint [19]. This investigation invites this discussion even in the perinatal state.

Investigations using animal models have demonstrated variable tissue concentrations of chemotherapy agents with fetal tissues containing 5–50% of maternal tissue concentrations—ovarian chemotherapy levels were not quantified [20]. Cytotoxic chemotherapy works by causing genetic damage to exposed cells including breaks, translocations, deletion, mutations, and cell cycle disruption [21]. Most organogenesis is complete by the end of the first trimester; however, the mitotic rate in oogonia peaks in the second trimester prior to widespread transition to meiosis and arrest in the diplotene stage [22]. Greater than half of oocytes are normally lost to regression and failure to be enveloped by granulosa cells. It is uncertain if this dynamic period of oogonial multiplication and oocyte formation may be more vulnerable to chemotherapy and if exposures during this window could result in an even great loss of the primordial reserve.

An ex vivo mouse model offers the ability to closely mimic maternal treatment level serum concentrations and directly evaluate the impact on oocyte number and cell death markers. At postnatal day 0 murine germ cell nests are present in the developing ovary and granulosa cells are just beginning to invade and encircle single oocytes [23]. This

postnatal murine process is similar to what occurs in human fetal ovaries in the late second to third trimester activity of the human ovary. Also, the use of a postnatal sample reduced the need for additional animal sacrifice by allowing mature female mice to remain in the breeding pool. The drug exposures planned were doxorubicin, cyclophosphamide, paclitaxel, docetaxel, and cisplatin. These five agents are among the most often utilized chemotherapy drugs for cancer treatment in reproductive age females per the National Comprehensive Cancer Network treatment algorithms.

We hypothesized that exposure to chemotherapy during the dynamic early period of oogonial multiplication and oocyte formation, as may occur during maternal chemotherapy in the second and early third trimester, would result in a significant decrease in the primordial reserve as measured by density of germ cells that varies in magnitude by chemotherapeutic agent.

2. Materials and Methods

2.1. Animals

All animal protocols were reviewed and approved by the Brown University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (protocol # 19-07-0001, November 2019).

Healthy wild type C57BL/6 mice were purchased from Jackson Labs and bred to produce neonatal pups. Mice were maintained on standard light: dark cycles with laboratory mouse chow provided ad libidum. All female pups on postnatal day (PND) 0 without milk spots were included in collection and randomization. Cages were checked daily, and day 0 newborn pups were collected and euthanized via decapitation. Neonatal ovaries were dissected with tissue forceps and syringe needle in sterilized phosphate-buffered saline (PBS) under a dissecting microscope under sterile conditions.

Ovaries were cultured as previously described [24,25] in 24-hanging-well-culture plates (Corning Transwell Polyester Inserts # CLS3470, Corning, NY, USA) with 330 μL of media. Ovarian culture media was pre-equilibrated (Weymouth's Media Life Technologies, Weymouth, MA, USA), 5% knockout serum replacement (Life Technologies), 10 μg/mL bovine serum albumin (Sigma, St. Louis, MO, USA), 1% insulin–transferrin–selenium (ITS-G; Life Technologies), 25 μg/mL ascorbic acid (Sigma), 2.5IU/mL follicle-stimulating hormone (FSH; Sigma), and 1X penicillin/streptomycin (Life Technologies). Plates were incubated at 37 °C for a total of 7 days in humified 21% O_2 room air.

Sister ovaries were randomized using a simple randomization protocol to control (DMSO alone) or drug-exposure groups. Blinding was not utilized due to the need for appropriate culture media mixing precautions and drug disposal. A total of 100 ovaries were utilized 8 per condition as well as 12 controls.

2.2. Chemotherapy Exposure

Chemotherapeutic agents were obtained from the National Institutes of Health National Cancer Institute Developmental Therapeutics Program (a division of the National Institutes of Health, U.S. Department of Health and Human Services) through the Approved Oncology Drugs Set. This program provides services and resources to the academic and private-sector research communities worldwide and supplied FDA-approved chemotherapeutics at 10mM in DMSO. Planned exposures utilized the most common chemotherapeutics used in treatment plans through the National Comprehensive Cancer Network for breast, ovarian, or cervical with a viable pregnancy: cyclophosphamide, doxorubicin, docetaxel, paclitaxel, and cisplatin [26].

Drug C_{MAX} was matched to published pharmacokinetic data regarding documented adult serum concentrations [27] and is described in Table 1. C_{MID} was half the dose of C_{MAX}. Ovaries were allowed to equilibrate in culture for 48 h, at which time the media was changed. The chemotherapy agent or control DMSO was added to the culture in a single exposure at 48 h or postnatal day 2. Media were then changed every 48 h thereafter. Whole

ovaries were collected for analysis at PND 4 or PND 7 as noted in Figure 2. As the entire ovary was less than 3 mm, partial collection was not feasible.

Table 1. Exposure Concentrations for Chemotherapy Exposures.

Agent	Clinical Dose	C_{MID} (µg/mL)	C_{MAX} (µg/mL)	Mechanism of Action
Cyclophosphamide	600 mg/m²	16.7	33.4	Alkylation of DNA that is not cell-cycle or phase specifics resulting in inhibition of DNA replication and transcription.
Doxorubicin	60 mg/m²	1.8	3.7	Inhibit RNA and DNA synthesis. Also, inhibiting topoisomerase II, causing inhibition of DNA repair.
Docetaxel	100 mg/m²	2.2	4.4	Disruption in the equilibrium of polymerization and depolymerization of microtubules causing abnormal cellular function and disruption of replication leading to apoptosis.
Paclitaxel	175 mg/m²	1.8	3.7	Disruption in the equilibrium of polymerization and depolymerization of microtubules causing abnormal cellular function and disruption of replication leading to apoptosis.
Cisplatin	80 mg/m² (ovary) 50 mg/m² (breast) 40 mg/m² (cervix)	2.6 3.5	4.3	Platinum binds DNA forming intra-stranded and inter-stranded crosslinks. This inhibits DNA replication and transcription.

Figure 2. Schematic timeline detailing tissue protocol.

2.3. Immunofluorescent Staining

Collected whole ovaries were fixed for 10 min in 1% formalin and, then, were embedded in optimal cutting temperature compound (Tissue-Tek, Torrence, CA, USA) for sectioning. Ovaries were serially sectioned at 8uM on a Shandon Cryostat onto glass slides and washed in 1X PBS containing 0.01% Triton-X (Sigma). Tissue sections were then incubated in blocking buffer (3% goat serum (Sigma), 1% bovine serum albumin (Sigma), and 0.01% Triton-X in 1X PBS) and stained by incubation with primary antibodies against Tra98 (1:100; Abcam, Waltham, MA, USA) and mouse vasa homolog (MVH; 1:100; Cell Signaling, Danvers, MA, USA) for germ cell counting. A secondary antibody-only control was included to compare background staining. Sections were further stained with DAPI to visualize nuclei and observed on a Nikon epifluorescence microscope. Images were analyzed on ImageJ [28]. Germ cell density was calculated by first counting total MVH-positive cells, presumed germ cells by cell marker per slide. Then, this number was divided by the measured ovarian tissue as noted by DAPI positive staining. This yielded a value of cells/mm², or the density of germ cells in the ovary, as previously described [29]. Each condition was duplicated a minimum of 8 times and each tissue sample was analyzed across 4 separate slides.

Samples were excluded from analysis if upon microscopy they were found to not reflect ovarian tissue but instead an area of stroma or fallopian tube. Similarly, if staining artifact confounded adequate measurement and cell counts, that sample was excluded.

2.4. Cell Death Staining

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is widely used for the detection of apoptotic events in tissue sections. The assay detects DNA fragmentation by nicking and can help identify cell damage specific for DNA fragmentation occurring in cells in late apoptosis [30].

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche, #11684795910, Indianapolis, IN, USA), per the manufacturer's instructions. Briefly, tissue sections were collected as described above with immunofluorescent staining and, then, treated with 0.1% Triton x-100 for 2 min on ice. Intracellular DNA fragments were, then, labeled by exposing the cells to TUNEL reaction mixture for 1 h at 37° C in a dark humidified incubator. Slides were, then, washed with PBS twice and analyzed on Nikon epifluorescence microscope at 20× magnification.

2.5. Statistical Analysis of Data

Initial work in establishing the ex vivo culture protocol yielded a mean of 570 oocytes/mm^2 with a standard deviation of 300. We hoped to evaluate a clinically significant decrease in oocyte density of 75% or more with any drug exposure condition. This percentile drop would correlate to the mean change from the average oocyte population in a human female at 25 (65,000) to the oocyte population seen in a human female at 35 (16,000) [31].

Planned exposures were the most common 5 chemotherapy agents used in pregnancy at serum max dose or 50%, to gauge dose response. Cisplatin is also clinically used in 3 dosing strategies, so we utilized a third group here. This gave 11 exposure groups, with corresponding controls. With planning for an alpha of 0.05, beta of 0.2 with power of 0.8, a minimum of 8 samples per group or 88 samples would be needed.

All initial data are presented with descriptive statistics with mean and standard error. All data are presented as mean (95% confidence interval). A two-way ANOVA was performed using PRISM (GraphPad, Irvine, CA, USA). ANOVA was chosen as the collected data evaluated a quantitative dependent variable at multiple levels of two categorical independent variables. There were adequate observations in our dataset to be able to find the mean of the quantitative dependent variable at each combination of levels of the independent variables, and, as previously noted, these are presented along with their standard error. Values of $p < 0.05$ were considered statistically significant.

3. Results

The varying effects of chemotherapeutic exposure were quantified based on their immunofluorescent imaging. Representative images are seen in Figure 3 with oocytes being shown with dual markers of MVH and TRA98.
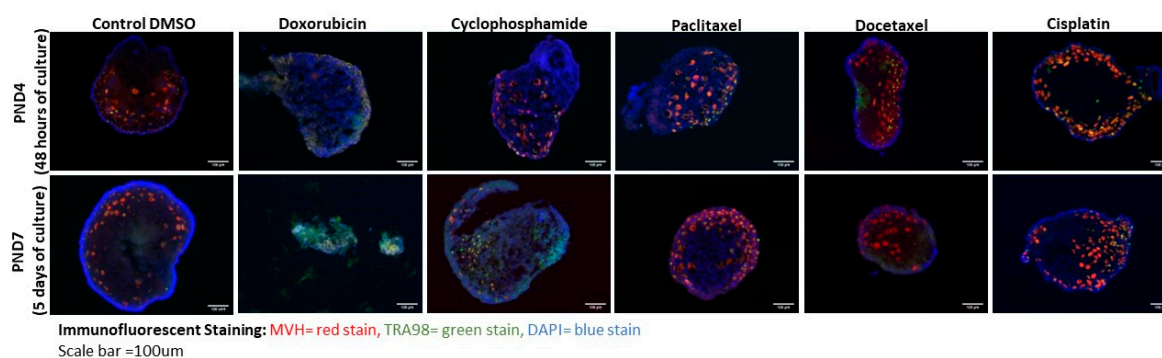

Immunofluorescent Staining: MVH= red stain, TRA98= green stain, DAPI= blue stain
Scale bar =100um

Figure 3. Immunofluorescent imaging demonstrating oocyte density across chemotherapeutic exposures.

3.1. Doxorubicin and Cyclophosphamide Exposure Result in Dramatic Loss of Oocytes in Culture

As noted in Table 2, oocyte density was first quantified after 48 h (PND 4) of exposure to control or serum matched doxorubicin. Control ovaries demonstrate a density of 693 oocytes/mm^2 with a standard error (SE) of 62 at 48 h of exposure. Quantitative review of samples showed loss of stroma and oocytes in doxorubicin exposed samples at the serum mid and max dose at 48 h, C_{MID} oocyte density of 21 (0–52) oocytes/mm^2 and C_{MAX} oocyte density of 63 (0–128) oocytes/mm^2. This represents a mean loss of 97% of oocyte density at C_{MID} and a loss of 91% at C_{MAX}.

Table 2. Oocyte density 48 h (PND4) and 120 h (PND7) following experimental exposure.

Condition	Oocyte Density at PND4 (% of Control)	Oocyte Density at PND7 (% of Control)	Adjusted *p* Value Two-Way ANOVA
Control (1 µL DMSO)	693 oocytes/mm^2 StErr = 62 (--)	570 oocytes/mm^2 StErr = 71 (--)	n/a
Doxorubicin Mid dose (1.83 µg/ml)	21 oocytes/mm^2 StErr = 16 (3%)	23 oocytes/mm^2 StErr = 9 (4%)	<0.0001
Doxorubicin Max Dose (3.66 µg/ml)	63 oocytes/mm^2 StErr = 33 (4%)	27 oocytes/mm^2 StErr = 8 (5%)	<0.0001
Cyclophosphamide Mid dose (16.704 µg/ml)	586 oocytes/mm^2 StErr = 120 (85%)	366 oocytes/mm^2 StErr = 29 (64%)	0.35
Cyclophosphamide Max Dose (33.408 µg/ml)	438 oocytes/mm^2 StErr = 45 (63%)	282 oocytes/mm^2 StErr = 8 (49%)	0.0004
Paclitaxel Mid dose (1.825 µg/ml)	628 oocytes/mm^2 StErr = 50 (91%)	796 oocytes/mm^2 StErr = 115 (140%)	0.7024
Paclitaxel Max Dose (3.65 µg/ml)	668 oocytes/mm^2 StErr = 27 (96%)	718 oocytes/mm^2 StErr = 98 (126%)	0.8157
Docetaxel Mid dose (2.21 µg/ml)	353 oocytes/mm^2 StErr = 2 (51%)	531 oocytes/mm^2 StErr = 96 (93%)	0.2794
Docetaxel Max Dose (4.42 µg/ml)	538 oocytes/mm^2 StErr = 173 (78%)	624 oocytes/mm^2 StErr = 61 (109%)	0.8616
Cisplatin: Low dose (2.593 µg/ml)	975 oocytes/mm^2 StErr = 120 (51%)	608 oocytes/mm^2 StErr = 52 (93%)	0.326
Cisplatin: Mid dose (3.457 µg/ml)	663 oocytes/mm^2 StErr = 29 (96%)	591 oocytes/mm^2 StErr = 27 (104%)	>0.9999
Cisplatin: Max Dose (4.321 µg/ml)	736 oocytes/mm^2 StErr = 151 (106%)	524 oocytes/mm^2 StErr = 80 (92%)	>0.9999

Media was changed after 48 h of drug exposure and specimens were cultured in routine media for an additional 3 days to reach 120 h following exposure (PND7). At this point uniform loss has appeared among C_{MID} and C_{MAX}. PND 7 controls demonstrate a density of 570 oocytes/mm^2 with a confidence interval of 431–709. Doxorubicin exposed PND 7 samples at the serum mid and max dose had a C_{MID} oocyte density of 23 (5–41) oocytes/mm^2 and C_{MAX} oocyte density of 27 (11–43) oocytes/mm^2. This represents a mean loss of 96% of oocyte density at C_{MID} and a loss of 95% at C_{MAX}. A two-way ANOVA confirms the significance of this relationship ($p < 0.0001$) at both concentrations.

Exposure to cyclophosphamide resulted in less of a decrement. At 48 h of drug exposure cyclophosphamide exposed samples at the serum C_{MID} concentration have an oocyte density of 586 (351–821) oocytes/mm^2 and C_{MAX} oocyte density of 438 (350–526) oocytes/mm^2. This represents a non-significant mean loss of 15% of oocyte density at C_{MID} and a loss of 37% at C_{MAX}, compared to controls. Cyclophosphamide exposed PND 7 samples at the serum mid and max dose had a C_{MID} oocyte density of 366 (309–423) oocytes/mm^2 and C_{MAX} oocyte density of 282 (266–298) oocytes/mm^2. Two-way ANOVA demonstrated that the loss seen with serum max concentration was significant ($p = 0.0004$) with a loss of 51% oocyte density compared to control. The C_{MID} dose did not reach significance ($p = 0.35$).

3.2. Cisplatin, Docetaxel, and Paclitaxel Exposure Did Not Lead to Oocyte Loss

Treatment with cisplatin resulted in an average oocyte density remained similar to controls even at the highest clinical concentrations, 524 (367–680) oocytes/mm^2. This represents a loss of only 8% and the C_{MID} concentrations demonstrated no loss. However, a qualitative phenotypic change in the oocyte population with preservation of smaller, more peripheral cells consistent with germ cell nests was noted during analysis efforts.

Docetaxel and paclitaxel demonstrated no significant change in oocyte density compared to control. At PND 7 docetaxel exposed samples had an oocyte density of 531 (342–719) oocytes/mm^2 at C_{MID} and 624 (504–744) oocytes/mm^2 at C_{MAX}. Paclitaxel exposed PND 7 samples had a mean C_{MID} oocyte density of 796 (571–1021) oocytes/mm^2 and mean C_{MAX} oocyte density of 718(526–910) oocytes/mm^2. This indicated a non-significant change in oocyte density for docetaxel and paclitaxel.

3.3. Cell Death Analysis

Each chemotherapeutic exposure demonstrated its own unique phenotypic pattern in analysis and is displayed with representative images in Figure 4. Control condition ovaries at PND 4 and PND 7, there is evidence of rare TUNEL-positive cells, largely in the central space. This was more notable at PND 7 days than at PND 4.
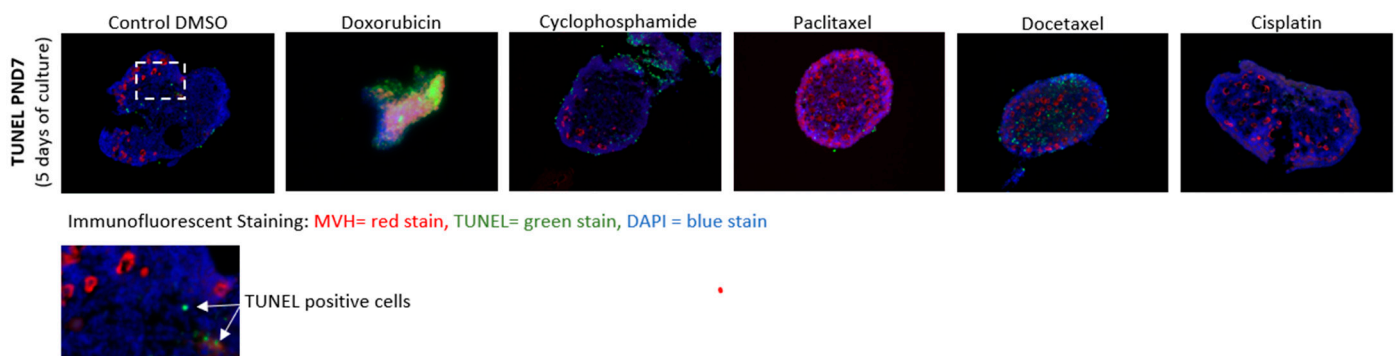

Figure 4. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining demonstrating cell death across chemotherapeutic exposures.

Doxorubicin treatment demonstrated near ubiquitous damage in germ and stromal cells with higher level of damage noted in stromal support cells after 24 h of exposure and effect seen in oocytes by 48 h of drug exposure. Cyclophosphamide treatment exhibited moderate TUNEL positivity; this largely localized to oocytes with pyknotic nuclei and enhancement of the central loss seen in control specimens. Stromal cells immediately ringing primordial oocytes were more affected following docetaxel exposure, and this effect seemed enhanced at day 7 over day 4. Paclitaxel exposure caused moderate TUNEL positivity, largely in stromal support cells but there was improvement between 4 and 7 days. Following exposure to cisplatin, the TUNEL imaging showed limited areas of concerns with rare cells effected on the periphery and no progression of damage.

4. Discussion

In female offspring, future fertility and fecundity can be compromised by any treatment that decreases the number of primordial follicle or alters functioning of the ovaries. Per the American Society of Clinical Oncology, the oncologist has a responsibility to inform patients about risks to future fertility [32], but there is scant data on transplacental exposure.

The unique exposure of in utero chemotherapy may result in profound premature ovarian insufficiency. Most cytotoxic drugs will cross the placenta given their low molecular weight [33]. Our ex vivo murine model suggests that the choice of chemotherapy during pregnancy may greatly impact future ovarian function and consideration should be given to selecting the least harmful chemotherapy regimen to ensure fetal health. By avoiding or delaying doxorubicin and cyclophosphamide, the delicate establishment of ovarian reserve in the fetus may continue even as the mother pursues lifesaving chemotherapy treatment.

During establishment of the ovarian reserve, oocytes may have unique cell-stage vulnerability that results in a distinct effect from that seen in reproductive age females. Cyclophosphamide, as an alkylating agent, resulted in significant loss of oocytes. This was consistent with previous clinical studies demonstrating detrimental effects on ovarian reserve with cyclophosphamide [32]. The adult clinical literature does not demonstrate a similar level of concern with doxorubicin. However, it was in these specimens that the greatest loss of oocytes was seen. This anthracycline caused a significant loss of ovarian reserve with a distinct pattern with rapid cell death in germ and stromal cells on TUNEL assay. Paclitaxel and docetaxel in our model had no significant effect on oocyte density. Cisplatin, uniquely, enhanced the overall analyzed oocyte density but was accompanied by a phenotypic change of small, bunched peripheral oocytes. It is uncertain whether longer-term culture or additional exposure would lead to apoptosis or simply delay.

This pivotal ex vivo work suggests unique enhanced sensitivities in immediately post-meiotic arrest oocytes. Cyclophosphamide is an alkylating agent that forms irreversible covalent bonds with amino, carboxyl, and phosphate groups in DNA, RNA, and proteins [34]. These inter- and intra-strand crosslinking in DNA reduces replication, and the actions of cyclophosphamide are not cell cycle-specific, but effects are enhanced in tissues with ongoing cellular proliferation. Doxorubicin is also not considered cell cycle-specific, but this antitumor antibiotic intercalates in DNA forming free radical intermediates which then results in DNA damage [35]. Doxorubicin can also inhibit topoisomerase [34]. It is unclear which of these mechanisms is the mechanistic cause of the dramatic oocyte damage seen in our samples. Cisplatin, as a platinum compound, also acts on DNA covalently bonding to cause intra- and inter-strand DNA adducts as well as additional effects with the intrinsic mitochondrial pathway [36]. Perhaps not surprisingly, the mitotic inhibition seen with exposure to docetaxel and paclitaxel seemed to cause little apoptosis in early oocytes in culture. The taxanes bind and stabilize microtubules—which are not in use in post-meiotic arrest oocytes [37]. But this detriment to microtubular action may have resulted in the DNA damage seen in TUNEL staining within the granulosa and stromal cells.

Further work in vivo and with human cohorts is needed to confirm these findings as well as explore other chemotherapeutic agents.

Limitations do exist for this ex vivo mouse model. While it offers tight control of chemotherapy concentration, it does not account for filtration and modification by the placenta, level of binding to plasma proteins, or for biotransformation resulting from hepatic metabolism. Longer-term cultures may also yield different results.

5. Impact on Clinical Practice

As doxorubicin is the most frequently used chemotherapy for malignancy in pregnancy [38], its gonadal toxicity raises significant questions as to the long-term impact on postnatal ovarian reserve. Treatment during pregnancy must balance the diagnosis, maternal health, and fetal health. Consideration might be given to tailoring a mother's chemotherapy regimen to protect her daughter's ability to have a normal pubertal transi-

tion and reproductive lifespan. Our model provides an outstanding template for assessing the impact of chemotherapeutic agents on oocyte development.

6. Future Directions

We have begun in vivo animal studies to gain further appreciation of the degree that placental filtration alters outcomes. A murine animal model offers the benefit of rapid evaluation of outcomes utilizing ovarian histology. Breeding studies following prenatal chemotherapy exposure may also offer further insight into fecundity and reproductive fitness.

Human studies may have significant time lag. Clinical trials tracking children born after chemotherapy exposure would offer additional future information. To date, a single cohort reports the birth of 12 s-generation children to individuals born after exposure to chemotherapy during their own gestation [12]. However, the rate of normal pubarche, menstruation or fertility is not discussed in the manuscript. Murphy et al. published their single-arm study of outcomes following chemotherapy in pregnancy that began in 1992 [39]. The oldest of this cohort is entering into child-bearing age now and could also be followed for reproductive health outcomes.

Continued scientific attention to this unique population is important as incidence of cancer associated with pregnancy is likely to continue to increase as more people delay childbearing. Reproductive health is part of the long-term survivorship goals not only for these adult women but also their daughters.

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