The Effects of Chemotherapy Treatment Schedules on Ovarian Function

By

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ABSTRACT

THE EFFECTS OF CHEMOTHERAPY TREATMENT SCHEDULES ON OVARIAN FUNCTION

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University of Guelph, 2014

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Chemotherapy can cause early menopause or infertility in women and have a profound negative impact on the quality of life of young female cancer survivors. Various factors are known to influence the risk of chemotherapy-induced ovarian failure, including the drug dose and treatment duration; however, the scheduling of dose administration has not yet been evaluated as an independent risk factor. We hypothesized that Low-Dose Metronomic (LDM) chemotherapy scheduling would be less detrimental to ovarian function than the traditional Maximum-Tolerated Dose (MTD) strategy. Treatments of MTD cyclophosphamide induced high levels of follicle atresia and enhanced follicle recruitment in mice. In contrast, LDM delivery of an equivalent dose of cyclophosphamide reduced growing follicle numbers, but was not associated with higher levels of follicle atresia or recruitment. Our findings suggest that LDM scheduling could potentially minimize the long-term effects of cyclophosphamide on female fertility by preventing follicle depletion from enhanced activation.
ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere appreciation to my advisor, Dr. Jim Petrik, for giving me the opportunity to take on this project. I am truly grateful for his unwavering patience, guidance and support, and the many invaluable experiences that I have had during my time in his lab. I would also like to thank the additional member of my advisory committee, Dr. Jon LaMarre, for his helpful insight and enthusiasm for this project.

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Special thanks to my family for their love and support, and to members of the Rowe family for their thoughtfulness and encouragement.

This thesis is dedicated to my best friend, Sam Rowe.
DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in the body of this thesis was performed by me.

Some intraperitoneal chemotherapy injections were performed by Samantha Russell. Helen Coates generously serial sectioned all formalin-fixed, paraffin-embedded ovaries used for follicle quantification and immunohistochemical analysis. Helen Coates also performed coverslipping of some sections. Blood vessel density was determined by Dr. Jim Petrik using images of cryosections fluorescently stained for CD31.

The SIGC cell line used for the in vitro model was provided by Dr. R. Burghart of Texas A&M University.
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<th>Full Form</th>
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<tbody>
<tr>
<td>4HC</td>
<td>4-Hydro(pero)xycyclophosphamide</td>
</tr>
<tr>
<td>ABAM</td>
<td>Antibiotic-antimycotic</td>
</tr>
<tr>
<td>ABL</td>
<td>Abelson murine leukemia viral oncogene homolog 1</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-Müllerian hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOF</td>
<td>Acute ovarian failure</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma gene-2</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region protein</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCNS</td>
<td>Cell cycle non-specific</td>
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<tr>
<td>CCS</td>
<td>Cell cycle specific</td>
</tr>
<tr>
<td>CD31</td>
<td>Cluster of differentiation 31</td>
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<tr>
<td>CD34</td>
<td>Cluster of differentiation 34</td>
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<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
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<tr>
<td>CEP</td>
<td>Circulating endothelial progenitor</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
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<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break (DNA)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>dUTP</td>
<td>2´-deoxyuridine, 5´-triphasate</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ETOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Flk-1</td>
<td>Fetal liver kinase 1</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
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<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HRP</td>
<td>Horseradish-peroxidase</td>
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<td>HSC</td>
<td>Haematopoetic stem cell</td>
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<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
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<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LDM</td>
<td>Low-dose metronomic</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LHR</td>
<td>Luteinizing hormone receptor</td>
</tr>
<tr>
<td>M</td>
<td>Mouse</td>
</tr>
<tr>
<td>MIS</td>
<td>Müllerian inhibiting substance</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum-tolerated dose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>NaV</td>
<td>Sodium orthovanadate</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>P4</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidyl inositol kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonic fluoride</td>
</tr>
<tr>
<td>PMSG</td>
<td>Pregnant mare serum gonadotropin</td>
</tr>
<tr>
<td>POF</td>
<td>Premature ovarian failure</td>
</tr>
<tr>
<td>POI</td>
<td>Primary ovarian insufficiency</td>
</tr>
<tr>
<td>QOL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>R</td>
<td>Rabbit</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIGC</td>
<td>Spontaneously immortalized rat granulosa cell</td>
</tr>
<tr>
<td>SIP</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SM</td>
<td>Skim milk</td>
</tr>
<tr>
<td>T3</td>
<td>3, 5, 3’-triiodothyronine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP nick end labeling</td>
</tr>
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<td>V</td>
<td>Volts</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>Vascular endothelial growth factor receptor-1</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor-2</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WST-1</td>
<td>Water-soluble tetrazolium salt 1</td>
</tr>
<tr>
<td>ZPR</td>
<td>Zona pellucida remnant</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha smooth muscle actin</td>
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</table>
INTRODUCTION

The risk of iatrogenic infertility for females undergoing chemotherapy prior to the age of menopause is estimated to be in the range of 30 to 70% (Blumenfeld et al., 2012). This broad approximation of risk is a reflection of a variety of contributing factors, including the age of the patient, as well as a number of treatment-related factors, such as the drug type used, the dose received, and the number of treatment cycles endured; each of which can be variable depending the patient’s circumstances and response to therapy.

Conventional chemotherapy is delivered at the patient’s maximum tolerated dose (MTD) and is followed by a drug-free interval to allow recovery from the toxic side-effects of the drug before initiation of the next cycle. Historically this has been considered the most effective scheduling approach with respect to tumor cell kill. However, the recent discovery that traditional chemotherapeutic agents can exert anti-angiogenic effects when delivered at frequent small doses has changed the way many cancer therapies are being developed today. Preclinical applications of this new dosing strategy, known as low-dose metronomic (LDM) chemotherapy, have contributed to improved survival outcomes in a number of cancer models when used in combination with various anti-angiogenic drugs (Russell et al., 2014).

Many of these promising LDM treatments have advanced to clinical trials and have proven to be beneficial in a number of different applications, demonstrating clinical efficacy along with little to no short-term side-effects. As a newly introduced treatment treatment strategy, the long-term effects of LDM scheduling are still unknown. This includes its impact on female fertility, which may take years or even decades after the exposure to chemotherapy to become apparent.

REVIEW OF LITERATURE

Cancer and Chemotherapy

Current estimates suggest that 2 in 5 Canadians will be diagnosed with cancer in their lifetime (Canadian Cancer Statistics, 2014). While the incidence of cancer continues to increase, fortunately cancer survivorship is also on the rise thanks to improved detection and treatment protocols. Cancer treatment strategies are specifically tailored to each individual patient, taking into consideration a number of factors including the type, stage and location of the cancer, the goal of treatment, as well as the patient’s state of health and preference. Typically, each regimen will involve various combinations of local, systemic and targeted therapies. Systemic treatment by the use of chemotherapeutic agents is one of the most commonly applied cancer treatment strategies as it can be beneficial in a number of clinical settings. Chemotherapy is used as
primary treatment for non-solid tumors, such as hematological malignancies or metastatic disease (primary induction chemotherapy). For the treatment of solid tumors or localized disease it may be used prior to surgery or radiation in attempt to minimize tumor burden (neoadjuvent therapy). Furthermore, chemotherapy can be used as follow-up to such treatments to prevent metastasis or disease recurrence from possible remaining or disseminated tumor cells (adjuvant therapy). Chemotherapeutics are frequently administered in combination with additional chemotherapy drugs as well as other targeted therapies. Combining drugs with varying cytotoxic mechanisms increases the number of cellular targets, enhances tumor susceptibility and potentially prevents or attenuates resistance to treatment.

**Chemotherapy Scheduling of Administration**

Since the development of chemotherapeutics, great efforts have been made to maximize their anti-tumor effects. Tumors respond to chemotherapy in a dose-dependent manner; however, the associated toxicity of these types of drugs prevents the use of maximally effective doses. Over the years, researchers have explored various chemotherapy schedules (i.e. treatment regimens with respect to drug dose and frequency of administration), balancing the ratio of drug efficacy to toxicity in attempt to optimize treatment success.

Conventional chemotherapy scheduling, referred to as maximum-tolerated dose (MTD) chemotherapy, involves delivery of extremely high doses of cytotoxic agents over a brief administration period. Skipper and associates provided the hypothetical groundwork for this chemotherapy strategy that is still being used today. Using a murine L1210 leukemia model, they demonstrated that the effects of cytotoxic drugs follow log cell-kill kinetics. That is, a given dose of chemotherapy will destroy a constant fraction of cancer cells rather than an absolute number (Skipper et al., 1964). Based on the observation that the malignant cells in this model have an exponential or logarithmic growth pattern, they proposed that if sufficient drug is administered so that the proportion of tumor cells destroyed exceeds re-growth before the next treatment, then tumor burden can be reduced until the cancer is ultimately cured.

This concept led to the aggressive use of chemotherapeutics at intermittent schedules. Although, unfortunately, high doses of chemotherapy are associated with near life-threatening side-effects due to their indiscriminate action toward rapidly dividing cells. Normally proliferative healthy cells of the body, such as those in the bone marrow, gastrointestinal and reproductive tracts, and hair follicles can also be targets for the majority of chemotherapy drugs. With common side effects of neutropenia, neuropathy, nausea, vomiting, hair loss and mucocitis, this type of treatment can be extremely difficult for cancer patients to endure. Bone marrow transplants and the administration of hematopoietic growth factors (granulocyte-colony stimulating factors) are common strategies used to overcome bone marrow suppression, the major dose-limiting factor.
for most chemotherapy drugs, so that even higher doses can be administered. Facing the highest possible tolerable doses, patients require a period of rest in order to recover from the harsh drug effects before they are able to withstand the next treatment. As a result, MTD chemotherapy is administered in cycles that must include a rest period of at least 2 to 3 weeks, which provides the tumor and its supporting vasculature the opportunity to re-grow (Bertolini et al., 2003).

The log-kill hypothesis assumes that tumor cell kill by chemotherapy is proportional regardless of tumor cell burden. While this may hold true for non-solid tumors, such as leukemia, it does not accurately model solid tumors which do not exhibit constant growth rates. In 1976, scientists Norton and Simon discovered that small tumors grow at a faster rate than large tumors and could be better modeled by the Gompertzian growth curve (Norton et al., 1976). Furthermore, they demonstrated that tumor regression rates in response to chemotherapy are proportional to the rate of tumor growth at the time of treatment. Based on these findings, which suggested that smaller tumors should be easier to treat, they argued that it would be more beneficial to make treatments more “dose-dense” by reducing the time interval between treatments. This concept was often referred to as the Norton-Simon Hypothesis (Simon and Norton, 2006), but it was not until decades later that dose-dense regimens were finally tested in clinical trials. In 2003, efficacy of a traditional 3-week cycle was compared to that of a shortened 2-week cycle (with the administration of granulocyte-colony stimulating factor) for the adjuvant treatment of node-positive primary breast cancer. In this study, the 2-week dose-dense regimen had significantly improved disease-free and overall survival (Citron et al., 2003). In addition to the use of growth factors, treatment regimens can be made more dose-dense by gradually exposing the patient to elevated doses of cytotoxic drugs (dose-escalation) or by strategically scheduling drug combinations in a way that minimizes toxicity overlap (concurrent versus sequential administration).

Overall, the aggressive application of chemotherapeutics combined with various other cancer treatment strategies has contributed to improved survival rates in patients with solid tumors and is even considered curative for a list of non-solid tumors including, but not restricted to various types of lymphomas and leukemias, germ cell cancer, and small cell lung cancer (Loven et al., 2012). Treatment success is limited, however, by drug toxicity and the development of drug resistance in a large fraction of patients. Fortunately, there is now accumulating evidence to suggest that these limitations may be challenged by a newly emerging treatment strategy known as low-dose metronomic (LDM) chemotherapy. This scheduling approach involves more frequent (daily to weekly) administration of substantially lower chemotherapy doses, which reduces or eliminates the drug-free interval and maintains constant chemotherapeutic pressure on the cancer cells. The LDM approach is based on the recent discovery that when delivered by continuous low doses, conventional chemotherapeutics exert enhanced anti-angiogenic effects. In the year 2000, it was demonstrated that mice with tumors considered resistant to (MTD)
cyclophosphamide displayed considerable sensitivity to the drug when lower doses were administered at shorter time intervals and that endothelial cell apoptosis preceded tumor cell death in these animals (Browder et al., 2000). In a related study, continuous low dose administration of vinblastine in combination with an anti-angiogenic agent resulted in sustained tumor regression in mice bearing human neuroblastoma xenografts (Klement et al., 2000). In both cases, LDM scheduling was found to be more effective in suppressing tumor growth than MTD scheduling and there were no signs of LDM drug resistance over the course of treatment.

A number of potential mechanisms are believed to contribute to the anti-angiogenic, and consequently, the anti-tumor effects of LDM chemotherapy. Thrombospondin-1 (TSP-1) is expected to play a major role in mediating the anti-angiogenic effects of LDM scheduling of chemotherapy agents. Continuous low dose exposure to various classes of cytotoxic drugs in vitro leads to increased expression of this endogenous anti-angiogenic factor (Bocci et al., 2003). Additionally, the anti-angiogenic and anti-tumor effects of cyclophosphamide are diminished in TSP-1 null mice (Bocci et al., 2003). Upon binding to the endothelial cell receptor CD36, TSP-1 blocks endothelial cell proliferation and induces apoptosis (Jiménez et al., 2007). Thrombospondin-1 may also inhibit angiogenesis by binding and sequestering a potent pro-angiogenic factor known as vascular endothelial growth factor (VEGF) (Gupta et al., 1999). Low-dose metronomic chemotherapy prevents the regrowth of blood vessels by suppressing the mobilization and viability of bone marrow-derived circulating endothelial progenitors (CEPs) (Bertolini et al., 2003). The opposite effect is observed in response to MTD chemotherapy scheduling, and the resultant elevated levels of CEPs are thought to contribute to the rapid recovery of the tumor vasculature between successive MTD treatments (Lynden et al., 2001; Bertolini et al., 2003). Furthermore, there is evidence that LDM chemotherapy exerts immunomodulatory effects that contribute to tumor reduction. Increased recruitment of natural killer (NK) cells, macrophages and dendritic cells is thought to contribute to the regression of brain tumor xenografts following LDM chemotherapy in mice (Doloff et al., 2012). In addition to having a direct dose-dependent effect on tumor cells, this scheduling approach may provide a possible mechanism for targeting a subpopulation of cells believed to be cancer stem cells (CSCs) (Folkins et al., 2007; Martin Padura et al., 2012).

Although we are currently awaiting definitive results from phase III clinical trials, LDM chemotherapy has been associated with promising anti-tumor effects, as well as favourable toxicity profiles in a number of completed trials (Loven et al., 2013; Lien et al., 2013). Consistent with what was demonstrated in mice with cyclophosphamide (Browder et al., 2000), a recent trial revealed that patients no longer sensitive to MTD administration of capecitabine respond to LDM scheduling of the drug (Fedele et al., 2012). This provides rationale for the use of LDM therapy as a possible rescue therapy. Various LDM treatment regimens have progressed to phase III clinical
trials despite the fact that the majority of patients enrolled in these trials are elderly or frail, or have already been heavily pre-treated and developed resistance to MTD therapy. At this time we are awaiting definitive results from ongoing clinical phase III trials, in which LDM scheduling is being applied to the following clinical settings: as consolidation treatment following conventional treatment where there is a high risk of relapse, as palliative care in the case of advanced stage disease, and as maintenance therapy as a temporary substitute for MTD chemotherapy (Loven et al., 2013). There are also a number of trials currently evaluating the efficacy of LDM chemotherapy combined with targeted anti-angiogenic agents, since the pairing of these treatment strategies has been associated with additive or synergistic drug effects in a number of preclinical studies (Bello et al., 2001; Loven et al., 2010; Russell et al., 2014). Optimal anti-angiogenic treatment doses and drug combinations, and the long-term efficacy of LDM chemotherapy need to be further addressed in order to validate its potential application as an alternative scheduling approach to MTD therapy (Loven et al., 2013; Lien et al., 2013).

Chemotherapy-Induced Female Infertility

Enhanced detection along with continually evolving treatment strategies are contributing to improved long-term survival rates for most cancers. As a result, chemotherapy-induced infertility is becoming a growing concern for women of reproductive age (Reh et al., 2011). Chemotherapy can cause irreversible ovarian damage that puts females aged 0 to 50 at risk of premature ovarian failure. In Canada, women under the age of 50 are estimated to make up 7% of all new cancer diagnoses in 2014 (Canadian Cancer Statistics, 2014). Fortunately, the vast majority of cancers common to this population are currently associated with relatively high survival rates. For example, breast cancer accounts for approximately 32% of these cases, which now has a 5-year survival rate approaching 90% - a 40% increase since the mid-80's (Canadian Cancer Statistics 2014). Mortality rates for breast cancer are decreasing at a rate of about 2.2% each year and this is largely due to increased mammography screening and the development of more effective post-surgery therapies (Canadian Cancer Statistics, 2012). Furthermore, advances in the treatment of childhood malignancies (ages 0 to 14) have contributed to a combined survival rate of 83% (Statistics Canada, 2014). Due to increased cancer diagnoses along with improved chances of survival, there is a continually growing population of young female cancer survivors. Therefore, it is becoming increasingly important to address concerns about the long-term effects of treatment and explore potential fertility preservation options for females undergoing chemotherapy.

Women that receive chemotherapy may experience both short and long-term ovarian damage that can result in a narrowing of their reproductive window. Temporary amenorrhea is common a common side-effect that immediately follows chemotherapy treatment that is a sign of
destruction growing follicles. This is clinically known as acute ovarian failure (AOF), since menses may resume over time if sufficient primordial follicles remain to replenish the maturing follicle population. The symptoms of irreversible long-term damage, which are indicative of near exhaustion of the limited primordial follicle population, may not emerge until years or even decades after treatment. Loss of ovarian function prior to the age of 40 is often referred to as premature ovarian failure (POF), primary ovarian insufficiency (POI) or early menopause. Considering the average age of first-time mothers in Canada is now over 29 years (Milan, 2008), and the fact that women naturally experience a drastic decline in fertility in the 10 years leading up to menopause (O’Conner et al., 1998), women that have undergone chemotherapy may experience greatly reduced opportunity to conceive, even if menses resumes after chemotherapy. In addition, women are advised to prevent pregnancy for two years post-treatment to due to the possibility of disease recurrence.

Studies have shown that cancer treatment-associated infertility can have a profound negative impact on the quality of life (QOL) of female cancer survivors. In a recent survey, women requiring chemotherapy were asked to rate their level concern about the impact of cancer treatment on fertility and the importance of having a child in their life. Based on responses from premenopausal women, both questions received an average score of 6.1 out of 7, with 7 being the highest possible rating of concern or importance, respectively (Reh et al., 2011). Women with treatment-related infertility were also more likely to report poorer mental health, and physical and psychological well-being than those without reproductive problems (Wenzel et al., 2005). Providing specialized counseling about reproductive loss and fertility preservation options prior to chemotherapy has been shown to improve the QOL of cancer survivors and contribute to reduced feelings of regret (Letourneau et al., 2012).

**Impact of Chemotherapy on Fertility and Associated Risk Factors**

In the literature, the risk of chemotherapy-induced infertility has been reported to be anywhere from 10 to 100%. For premenopausal women that undergo bone marrow transplantation following aggressive chemotherapy-conditioning protocols, the risk is increased to 90 to 100% (Carter et al., 2006). The overall average risk for standard treatments, however, is more likely in the range of 30 to 70% (Blumenfeld et al., 2012).

Differences in the measurement of fertility outcome may contribute, in part, to the wide range of estimated risk associated with chemotherapy. The definition of compromised ovarian function varies throughout the literature. The terms POF, POI and premature or early menopause are all loosely defined by the cessation of ovarian function or amenorrhea for a certain length of time prior to the age of 40 (Albright et al., 1942). Premature ovarian failure (POF) is clinically defined by at least 4 months of amenorrhea along with elevated follicle-stimulating hormone
(FSH) levels in women under the age of 40. Although the cessation of menses is considered a marker for early menopause (Partridge et al., 2007), using amenorrhea as an indicator of chemotherapy-induced reproductive damage grossly underestimates the true reduction of a woman's reproductive potential since infertility or subfertility may be experienced despite the presence regular menstrual cycles (Letourneau et al., 2012). Furthermore, increased FSH does not necessarily indicate irreversible ovarian function (Rebar et al., 1982). For these reasons, the term primary ovarian insufficiency (POI) is commonly believed to be a more accurate descriptor of compromised reproductive function (Albright et al., 1942). In addition to amenorrhea and FSH levels (Lowe et al., 1999; Del Mastro et al., 2011), hormonal measurements of estradiol (E2) (Reh et al., 2008; Tarumi et al., 2009; Del Mastro et al., 2011), progesterone (P4) (Badawy et al., 2009; Del Mastro et al., 2011), anti-Müllerian hormone (AMH) (Gerber et al., 2011) and inhibin B (Blumenfeld, 2005; Gerber et al., 2011) are also used in various combinations to predict reproductive outcome, though not all hormonal markers are equally representative of long-term ovarian damage. Of these potential fertility markers, it is widely accepted that AMH best correlates with follicle reserve (Fanchin et al., 2003; Grynnerup et al., 2012).

The combined use of radiation therapy, chemotherapy drugs and other biological targeted therapies makes it challenging to determine the gonadotoxic impact of chemotherapy in general or a particular cytotoxic agent on its own. It is also possible that estimation of risk associated with chemotherapy treatment regimens based on prospective or retrospective cohort studies do not accurately reflect current protocols. A 20-year-old woman undergoing chemotherapy today may not show signs of the long-term effects of treatment for over 10 years, and for childhood cancer survivors, the gap could be even greater. Standards treatments will likely change during this time with the addition of new drugs or drug combinations that improve survival outcome. Treatment regimens will also vary for each individual patient depending on a number of circumstances. For these reasons, it is important to identify independent risk factors and their impact on fertility. The type of chemotherapeutic agent(s) utilized, the dose administered and the duration of drug exposure are all treatment-related factors that have been determined to influence the severity of ovarian damage and the likelihood of infertility. A woman's age at treatment also influences the risk of infertility due to chemotherapy.

The alkylating agent class of chemotherapy drugs is associated with the highest risk of gonadotoxicity in women. This class includes drugs such as cyclophosphamide, ifosfamide, chlorambucil, procarbazine, melphalan, chloromethamine and busulfan. Platinum-based chemotherapeutics are considered medium-risk (cisplatin, carboplatin), followed by taxane mitotic inhibitors (paclitaxel, doxetaxel) and doxorubicin of the anthracyclin antibiotic class. Vinca plant alkaloids (vincristine, vinblastine), anthracyclin antibiotics (bleomycin) and antimetabolites (methotrexate, 5-fluorouracil, 6-mercaptopurine) are considered low-risk agents (Blumenfeld,
Combined, these drug toxicities contribute to the varying degrees of ovarian damage associated with different treatment regimens. For example, the MOPP-ABVD (chloromethamine, oncovin, procarbazine, and prednisolone combined with adriamycin bleomycin, vinblastine and dacarbazine) regimen is associated with a 50% risk of POF, whereas risk associated ABVD alone is less than 10% (Mackie et al., 1996; Behringer et al., 2005). The length of treatment is a major contributing factor as well. Two cycles of COPP (cyclophosphamide, oncovin, procarbazine and prednisolone)/ABVD for the treatment of Hodgkin’s lymphoma can lead to POF in approximately 4% of women under the age of 30, but four cycles of the same regimen will cause POF in 23.5% (Behringer et al., 2005). In addition, the magnitude and severity of chemotherapy-induced ovarian damage is dose-dependent. For instance, eight cycles of the standard BEACOPP (bleomycin, etoposide, adriamycin, cyclophosphamide, oncovin, procarbazine and prednisone) regimen compared to dose-escalated BEACOPP increases the risk of chemotherapy-induced amenorrhea from 32% to 67% (Behringer et al., 2005). These factors could also be considered together as the cumulative dose.

The cumulative doses of cyclophosphamide predicted to cause POF in women are 20 grams for 20-year-old patients, 9 grams at 30 years and 5 grams at 40 years (Blumenfeld, 2012). This also demonstrates that increased age at the time of chemotherapy is also associated with increased risk of future reproductive dysfunction. A large-scale cohort study of individuals diagnosed with cancer from 1953 to 2004 revealed that compared to their siblings, females treated with cyclophosphamide in their pediatric (age 0-14), adolescent (15-19) and adult (20-34) stages of life had a relative probability of parenting a first child of 0.67, 0.69 and 0.57, respectively (Madanat et al, 2008). Age at diagnosis positively correlates AOF and infertility rates following chemotherapy treatment for breast cancer, Hodgkin’s disease, non-Hodgkin’s lymphoma, gastrointestinal malignancies and lymphoma. This relationship was most pronounced in women treated for Hodgkin’s disease, who displayed a four to five-fold increase in the rate of infertility from ages 18 to 40 (Letourneau et al., 2012). Older women are also less likely to regain regular menses after chemotherapy (Petrek et al., 2006; Letourneau et al., 2012; Henry et al., 2014). This correlation is presumed to reflect the fact that older women already have reduced primordial follicle numbers at the time of treatment and are more likely to experience signs of exhaustion of the ovarian reserve. Conversely, recent studies have shown that the closer a woman is to the age of natural menopause, the less likely she is to experience early menopause if AOF does not occur immediately after treatment (Letourneau et al., 2012; Partridge et al., 2007). This may be indicative of an age-related difference in primordial follicle sensitivity to chemotherapy that has not yet been explored, challenging the assumption that older primordial follicles are more susceptible to the toxic effects of the drugs.

The scheduling of chemotherapy administration (MTD versus LDM chemotherapy) has
not yet been identified as an independent risk factor of chemotherapy-induced infertility.

**Normal Ovarian Function**

**Ovarian Follicle Development**

The ovary functions to support the maturation and release of viable female gametes for fertilization and the production of steroid and protein hormones that are essential for female physical development and reproduction. This is achieved through a process known as follicle development, or folliculogenesis, which involves growth and maturation of an oocyte, and the proliferation and differentiation of its surrounding somatic cells to the point of release of the mature oocyte into the female reproductive tract.

Within each ovary is a finite and continuously depleting population of oocytes arrested in the first prophase of meiosis (primary oocytes). Each oocyte is invested by a single, flat layer of pregranulosa cells, and these cellular units collectively make up the primordial follicle. Primordial follicles can be maintained in a dormant state for up to about 50 years, or until menopause is reached. Periodically, a cohort of primordial follicles is recruited from this resting population to the developing follicle population upon activation of the oocyte. Following recruitment, the primordial follicle will transition into a primary follicle, which is characterized by a single layer of cuboidal granulosa cells surrounding a now metabolically active oocyte. The granulosa cells will increase in number as the oocyte increases in size during the early stages of folliculogenesis.

Further classification of follicular maturation stages will vary in the literature depending on the author’s preference and species investigated; however, there are a number of defining events that are essential for the transition into a mature ovulatory follicle. These include the acquisition of a theca cell layer, vascularization and the appearance of a fluid filled antrum. Granulosa cells promote nearby stromal cells to differentiate into theca cells, which eventually form two distinct layers encompassing the granulosa cell layer. The innermost layer, the theca interna, remains separated from the granulosa cell layer by the basement membrane or basil lamina. As these cells continue to proliferate and the oocyte increases in diameter, the follicle can no longer rely on passive diffusion of oxygen and nutrients from stromal blood vessels. Therefore, the theca interna and externa layers of the follicle becomes microvascularized. Fluid filled cavities eventually form within the granulosa cell layer and coalesce into a single antrum. The follicular fluid within the antrum is made up of transudate from the thecal vasculature along with protein secretions, such as paracrine factors from the follicular cells, that will further modulate oocyte maturation and ovulation. Preovulatory follicles will have a single large antral space within the granulosa cell layer that creates defined mural (outermost) and cumulus (surrounding the oocyte) granulosa cell layers. Only a species specific number of selected dominant follicles are chosen
from each cohort to successfully become an ovulatory follicle, which will rupture and release the oocyte and cumulus cells outside of the ovarian surface epithelium, marking the end of folliculogenesis. The remaining, non-dominant or subordinate follicles degenerate by a process known as atresia. The regulation of ovarian follicle development and dominant follicle selection is briefly summarized in Figure 1.

**Figure 1: Regulation of ovarian follicle development.** A combination of follicle-secreted and oocyte derived factors control the activation of primordial follicles from a quiescent state. Factors that promote follicle activation are indicated by green arrows and those responsible for maintaining primordial follicle quiescence are indicated by red arrows. Follicle activation initiates gonadotropin-independent growth and the secretion of factors that control the size of the growing follicle population. By the early antral stage of development, follicles are responsive to gonadotropin hormone (FSH and LH) stimulation and are recruited for antral follicle growth. In response to gonadotropins, antral follicles secrete hormones such as estradiol. Elevated estradiol levels cause a surge of LH that triggers ovulation. After ovulation, the remaining follicular cells will undergo luteinization to form the corpus luteum; a temporary endocrine structure that continues to produces estradiol and progesterone to prepare the uterine endometrium for implantation. (Images obtained and modified from mcg.ustc.edu.cn/db/follado/index.php for follicle stages and www.sydneyivf.com for endocrine profiles).
Unlike the pre-antral stages, in which follicular development is primarily under intraovarian control (Hirshfield, 1991; Fortune 1994), further development into the antral stages of follicular growth and survival during this phase of growth is dependent on gonadotropin stimulation. The cyclic development of antral follicles, ovulation and the menstrual cycle are initiated at the onset of puberty when maturation of the hypothalamo-pituitary-gonadal axis leads to secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus. This stimulates the pulsatile release of gonadotrophins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. Follicle-stimulating hormone and LH primarily act on the granulosa and theca cells of gonadotropin-sensitive follicles, respectively. Elevated levels of circulating FSH beyond a critical threshold will recruit a cohort or wave of responsive follicles to the follicular phase in which they will undergo antral follicle development.

It has been well established that FSH is a critical factor in dominant follicle selection, which is thought to largely occur during the early to mid-follicular phase (Fortune, 1994). Dominant follicles are selected based on their ability to continue to mature in an environment that inhibits further development of the subordinate follicles. In response to gonadotropin stimulation, the developing follicle will secrete factors, such as E2 and inhibins, which in turn suppress FSH levels at the pituitary level. Follicles that are not able to adapt to declining FSH levels will regress and succumb to atresia, while superior differentiation of the dominant follicle(s) leads to increased FSH sensitivity and allows continued growth. The role of FSH in dominance selection is supported by the discovery that elevated FSH levels beyond the natural threshold will permit a greater number of dominant follicles to be selected in that cycle. In addition, sustained FSH levels allows the progression of multiple follicles to the preovulatory stage, rather than a single dominant follicle in women (Baird, 1987; Schippe et al., 1998). These findings have provided the basis for controlled ovarian hyperstimulation (superovulation) techniques used in assisted reproduction to enhance female fertility.

The actions of gonadotropin hormones fuel the production of local factors that promote an environment that only increases the FSH sensitivity of select follicles. Gonadotropin stimulation of theca and granulosa cells is necessary for their cooperative biosynthesis of E2 from cholesterol (Armstrong, 1979; Hillier et al., 1981), a process described by the two cell theory for ovarian steroidogenesis. In brief, androgen is synthesized from cholesterol in LH-stimulated theca cells, and after diffusion across the basement membrane, androgen is further transformed into E2 by granulosa cell aromatase activity under FSH stimulation. Dominant follicles secrete higher amounts of E2 (Hillier et al., 1994), making their follicular fluid primarily estrogenic, whereas the follicular fluid of non-dominant, atretic follicles is predominantly androgenic (Westergaard et al., 1986; van Dessel et al., 1996; Bodensteiner et al., 1996). Therefore, increased FSH-induced aromatase activity is a defining characteristic of selected follicles, which is also supported by the
fact that selected follicles have a higher number of gonadotropin receptors (Bodensteiner et al., 1996).

Several other important oocyte and follicle-derived factors are involved in granulosa cell proliferation, oocyte maturation and the modulation of FSH-induced follicle activity via autocrine and paracrine mechanisms. This includes factors such as activins, inhibins, follistatin, TGF-β, bone morphogenic protein (BMP) and insulin-like growth factors (IGFs), among others. The intricate balance among these local factors in the follicular environment can either enhance or attenuate gonadotropin stimulation. They collectively play a role in determining the fate of each follicle, although many of their specific functions are somewhat unclear. Anti-Müllerian Hormone (AMH), also referred to as Müllerian Inhibiting Substance (MIS), is produced by granulosa cells following their differentiation from squamous to cuboidal cells. Expression of AMH is highest in preantral follicles and gradually decreases with follicular growth (Anderson et al., 2010). This expression pattern emphasizes the important role of AMH in maintaining follicle quiescence at the early stages, and inhibiting FSH-dependent growth at the later stages, of follicle development (Durlinger et al., 2001).

Expression patterns of inhibins, activins and follistatin are also reflective of their contribution to the regulation of folliculogenesis. An orderly shift from an inhibin B/activin dominant follicular microenvironment to one that is dominantly inhibin A/follistatin is believed to be necessary for dominant follicle development in women (Schneyer et al., 2000). Inhibin B is secreted by granulosa cells of follicles recruited by FSH, and along with E2, suppresses the release of FSH from the pituitary (Fraser et al., 1999). Activin A is also secreted by granulosa cells. In vitro studies using cultured rat granulosa cells have suggested that activin A promotes follicular development by stimulating granulosa cell proliferation and differentiation (Li et al., 1995; Miró et al., 1996). In a paracrine manner, activin A has also been shown to inhibit LH-induced production of androgen in cultured human theca cells (Hillier et al., 1991a). The opposite effect was observed in the presence of inhibin A, which appears to enhance androgen production in these cells (Hillier et al., 1991b). In addition, the effects of activin A can be antagonized by activin-binding protein, follistatin (Li et al., 1995; Cataldo et al., 1994). All together, these autocrine and paracrine factors make up the inhibin-activin-follistatin axis that contributes to the regulation of ovarian follicle development, in which an inhibin B/activin dominant microenvironment generally promotes follicular atresia and an inhibin A/follistatin dominant microenvironment enhances follicle survival.

A combination of endocrine and intraovarian factors promote increased follicular E2 secretion from dominant follicles. Follicular stimulation by FSH eventually induces the appearance of LH receptors (LHR) on granulosa cells of dominant follicles, which were previously
restricted to the thecal cell layer (Zeleznik et al., 1974; Erickson et al., 1979). A shift in FSH to LH-dependency prepares the follicle for a surge in LH from the pituitary that is triggered by a positive feedback switch from heightened E2 levels. Elevated LH prepares mature follicles for ovulation by triggering cumulus expansion (cumulus cell dispersion and deposition of a mucoid intercellular matrix) and the expulsion of the cumulus-oocyte complex from the ovary. The resulting changes at the cumulus cell/oocyte interface promote further oocyte maturation and its resumption of meiosis (Mehlmann, 2005). Sustained LH levels are also necessary for granulosa cell luteinization and the formation of the corpus luteum. This transient endocrine structure will continue to produce E2 and P4, the latter being an essential hormone for the preparation of the uterine endometrium for implantation and for maintaining an early pregnancy.

Angiogenesis and the Role of Pro- and Anti-Angiogenic Factors in Folliculogenesis

Continual remodeling of the ovarian vasculature is essential to support the cyclic nature of ovarian follicle development. As the follicle grows, it can no longer rely on passive diffusion of oxygen and nutrients from the stromal vasculature. In addition, progression throughout the antral stages of follicle development is dependent on hormonal support and the circulation of follicle-derived steroids. Therefore, cyclic ovarian vascularization is necessary for progressive growth of the ovarian follicle and also plays an important role in dominant follicle selection.

The developing follicle acquires its own vasculature by a process known as angiogenesis, defined as the formation of new vessels from pre-existing vasculature. Angiogenesis is a highly regulated process that is achieved through a delicate balance of pro- and anti-angiogenic factors. Two concentric capillary networks are formed around the follicle, one within each theca layer, while the granulosa layer remains avascular throughout folliculogenesis. It is not until after ovulation that the vasculature crosses the basement membrane separating the theca and granulosa cell layers, while undergoing rapid proliferation in order to support the developing corpus luteum.

Vascular Endothelial Growth Factor (VEGF) is a potent pro-angiogenic growth factor that promotes endothelial cell proliferation, survival and migration (Cross and Claesson-Welsh, 2001). In ovarian follicles, VEGF emerges at the pre-antral stage and accumulates as the follicle develops (Barboni et al., 2000; Greenaway et al., 2004; Yang et al., 2007). Its differential expression within follicles at various stages of growth suggests that VEGF is coordinately involved in the regulation of folliculogenesis. This has been demonstrated by studies involving manipulation of VEGF expression. The inhibition of VEGF in primates severely compromises ovarian function as evidenced by a reduction in granulosa cell proliferation, increased follicle atresia and the absence of ovulatory follicles (Wulff et al., 2002). In addition to a pro-angiogenic effect, these observations may be related to an avascular effect on granulosa cells, since VEGF
has been found to enhance the survival of granulosa cells in pro-apoptotic in vitro conditions (Greenaway et al., 2004). In vivo experiments using rats have shown that VEGF stimulation results in increased primary and secondary ovarian follicle numbers (Danforth et al., 2003) and a greater number of dominant follicles which are also hypervascularized (Shimizu et al., 2007). It has been postulated that enhanced hormonal support, and thus follicular dominance, can be achieved by increased follicular blood vessel density (Zeleznik et al., 1981). In support of this hypothesis, dominant estrogen-active follicles display enhanced VEGF expression and superior vascularization relative to estrogen-inactive non-dominant follicles, independent of their size (Grazul-Bilska et al., 2007).

The effects of VEGF in the ovary are initiated upon binding tyrosine kinase family receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR). In the ovary, these VEGF receptors are present on granulosa and theca cells, and vascular endothelial cells of thecal blood vessels (Berisha et al., 2000; Greenaway et al., 2004). Expression of VEGFR-1 and VEGFR-2 on theca cells does not change significantly during follicle maturation in cattle (Berisha et al., 2000). In contrast, granulosa cell VEGFR-2 expression increases with follicle size in cattle (Greenaway et al., 2004) and in water buffalo (Babitha et al., 2013), while significant changes have not been measured in VEGFR-1 expression. Therefore, it is most likely that the avascular effects of VEGF are primarily mediated by VEGFR-2 binding. Since VEGFR-2 activation is also believed to be responsible for the pro-angiogenic effects of VEGF (Kanno et al., 2000), it appears that VEGFR-2 is principally responsible for mediating the effects of VEGF in the ovary. Inhibition of VEGFR-2 by administration of an anti-VEGFR-2 antibody in primates causes altered regulatory hormone levels as well as a drastic increase in the duration of the follicular phase (Zimmerman et al., 2002).

Playing an opposite, but arguably equally important role in follicular development, is anti-angiogenic factor Thrombospondin-1 (TSP-1). Thrombospondin-1 is an extracellular matrix glycoprotein that is active in a range of processes, including cellular growth and differentiation, inflammation and angiogenesis (Chen et al, 2000). During ovarian follicle development, TSP-1 displays a distinct expression pattern that is generally opposite that of VEGF. In rats, TSP-1 is detected in the granulosa and theca interna layers of preantral and early antral follicles and can also be detected during early development of the corpus luteum (Petrik et al., 2002). Thrombospondin-1 prevents angiogenesis and promotes apoptosis in preantral and early antral rat follicles in vitro (Garside et al., 2010). It also induces apoptosis in isolated granulosa cells in a dose-dependent manner, demonstrating an additional avascular role in follicle development (Garside et al., 2010). Expression of TSP-1 is inversely related to follicle size and VEGF expression in cattle (Greenaway et al., 2005) and primates (Thomas et al., 2008). The reciprocal regulatory interactions between TSP-1, VEGF and their receptors contribute to their inverse relationship and follicle progression. Knockdown of TSP-1 in vitro and in vivo leads to increased
expression of VEGF, which can be partly explained by the ability of TSP-1 to directly bind and internalize VEGF (Greenaway et al., 2007). Consequently, TSP-1-null mice exhibit a larger population of growing follicles, follicular hypervascularization (Greenaway et al., 2007), and drastically reduced litter sizes (Lawler et al., 1998).

The effects of TSP-1 in the ovary are initiated upon binding CD36 receptors found on granulosa and theca cells, in addition to endothelial cells. It was recently demonstrated in our lab that CD36 knockout mice share a similar reproductive phenotype with TSP-1 knockouts, which includes elevated ovarian VEGF and VEGFR-2 expression (Osz et al., 2014). This supports previous findings that TSP-1/CD36 interactions can interfere with VEGF/VEGFR-2 signaling as a consequence of co-clustering and the direct interaction of the two receptors (Zhang et al., 2009). In contrast, inhibition of VEGF does not directly alter TSP-1 expression, but has been found to decrease CD36 in primate ovaries (Thomas et al., 2008), further supporting a functional association between the CD36 and VEGF receptors. Together, the interactions among these vascular factors and their receptors establish a feedback loop responsible for ovarian angiogenesis and follicle maturation; VEGF promotes TSP-1 activity by up-regulating CD36 receptor, which in turn leads to downregulation of VEGF activity (Thomas et al., 2008).

**Ovarian Targets of Chemotherapy**

Based on the presence of both short and long-term symptoms of ovarian dysfunction, it is apparent that the loss of female reproductive potential as a consequence of chemotherapy can be due to destruction of growing follicles and/or depletion of the limited primordial follicle pool. The first hit, AOF or amenorrhea shortly after chemotherapy administration, is the result of growing follicle loss, while the second hit, POF, POI, or premature menopause, is reflective of early depletion of the primordial follicle pool. This long-term, irreversible damage may be the outcome of direct primordial follicle damage or an indirect consequence of growing follicle atresia. Current research is focused on elucidating the specific mechanisms involved chemotherapy-induced infertility by identifying susceptible follicle stages and specific cellular targets of common chemotherapeutics.

The acute loss of developing follicles directly influences the rate of primordial follicle recruitment to the growing follicle pool. Follicle activation is negatively regulated by factors secreted by growing follicles, such as AMH and TGF-β (Kim, 2012). Disruption of these inhibitory factors allows uncontrolled primordial follicle activation, which can be demonstrated by culturing ovarian tissue in serum-free media (Wandii et al., 2007). In addition to controlling the rate of follicle activation, this effect may also provide compensatory mechanism to conserve ovarian function in the case of increased follicle atresia. However, this short-term response translates into long-term damage due to enhanced depletion of the ovarian reserve. The follicle 'burnout' theory...
describes this effect as the primary mechanism of long-term ovarian damage due to cyclophosphamide exposure (Kalich-Philosoph et al., 2013).

A number of studies have focused on pinpointing the follicular targets of chemotherapy. Folliculogenesis is a process that takes over 370 days to complete which is equivalent to about 13 menstrual cycles, and at any particular time, the ovary contains follicles in all stages of development (Gougeon, 1986). Two separate studies performed on whole rat (Pettrillo et al., 2011) and mouse (Desmeules and Devine, 2006) ovaries cultured in vitro and treated with metabolites of cyclophosphamide had fewer primordial and primary follicles. Surviving primordial follicles show signs of basement membrane damage and secondary follicles have also been reported to be a potential target of cyclophosphamide exposure in vitro (Raz et al., 2002).

In contrast to in vitro observations, when comparing human ovaries obtained for cryopreservation before and after treatment with various chemotherapy regimens, Abir and associates counted drastically higher numbers of viable preantral follicles, but no antral follicles, in patients under the age of 20 that had been pretreated with chemotherapy. They also noted deterioration of primordial follicle quality in pretreated patients, characterized by abnormal granulosa cell nuclei, oocyte vacuolization and basement membrane damage (Abir et al., 2008). These results suggest a two-fold mechanism of primordial follicle loss: through increased primordial follicle activation as compensation for growing follicle loss, and by direct primordial follicle damage. Similar observations were made following in vivo administration of cyclophosphamide in mice (Kalich-Philosoph et al., 2013). High levels of follicle atresia were initially measured after drug exposure, and shortly after, the number of small growing follicle increased in ovaries of treated mice relative to untreated controls. Since these observations were indicative of enhanced primordial follicle recruitment, it was postulated that cyclophosphamide was capable of directly inducing direct follicle activation through disruption of the PI3K/PTEN/Akt regulatory pathway responsible for primordial follicle quiescence. Analysis of whole cell ovary lysates exposed to the drug revealed increased phosphorylation of key modulators of primordial follicle activation (Akt, mTOR, rpS6 and FOXO3A). In addition, co-treatment with immunomodulatory compound AS101, which is known to inhibit this pathway, attenuated follicle activation in this model (Kalich-Philosoph et al., 2013). Although it was proposed that DNA damage may activate this pathway, a specific mechanism for direct cyclophosphamide-induced follicle activation has not yet been demonstrated.

Granulosa cells are believed to be highly vulnerable to the toxic effects of chemotherapy due to their proliferative nature, the characteristic of malignant cells that is most often targeted. Granulosa cells of small follicles exhibit increased levels of DNA damage and apoptotic markers following doxorubicin treatment in mice (Ben-Aharon et al., 2010; Soleimani et al., 2011).
Apoptotic granulosa cells have also been observed in growing follicles of mice after in vivo administration of irinotecan (Utsunomiya et al., 2008) and cyclophosphamide (Kalich-Philosoph et al., 2013). In comparison, theca cells have received very little attention with respect to chemotherapy-induced toxicity. Theca cells differentiate from unspecialized mesenchymal cells in the ovarian stroma, as opposed to granulosa cells which proliferate from a smaller population. For this reason, these cells have been considered a less likely target for agents that primarily target mitotically active cells. However, they should not be disregarded as they are still capable of proliferation (Rao and Midgley, 1978), or at least their precursors are, and cell cycle non-specific (CCNS) chemotherapy drugs will also target cells at rest. Theca cells are an essential component of ovarian function as they are indispensible for follicular hormone production. The follicular vasculature is also confined to the theca layer, making these cells the first to receive exposure to cytotoxic drugs. One study demonstrated a dose-dependent decline in the level of hyperpolarization-activated cation channels in theca and granulosa cells as well as oocytes after treating mice with the alkylating agent cisplatin (Yeh et al., 2009). The decline in channel expression had been previously linked to reproductive aging; however, their functional significance in the ovary is currently unknown (Yeh et al., 2008).

Considerable research has focused on the possibility of direct toxicity to the oocyte, which is the single most important cell of the ovarian follicle. Therapeutic levels of doxorubicin destroy mouse oocytes in vitro (Perez et al., 1997; Bar-Joseph et al., 2010) and in vivo (Perez et al., 1997). Also, DNA double-strand breaks are detectable in oocytes of small follicles within cultured mouse ovarian tissue treated with cisplatin (Gonfloni et al., 2009), and phosphoramide mustard, the active metabolite of cyclophosphamide (Petrillo et al., 2011). It is uncertain whether the initial insult that leads to follicle loss is related to oocyte or granulosa cell damage, as the bidirectional communication and support between these cell types is necessary for either one to survive (Kidder and Vanderhyden, 2010). Two similar studies examined the timing of apoptosis in oocytes and granulosa cells by xenografting human ovarian tissue that had previously been exposed to alkylating agents into immunodeficient mice (Oktem et al., 2007; Abir et al., 2008). Although one study used fetal and the other used adult human ovarian tissue, both models demonstrated that apoptotic cell death initially occurred in the oocyte. Conversely, mouse ovaries following doxorubicin treatment display the opposite order of events (Ben-Aharon et al., 2010). These dissimilar findings may reflect the different mechanisms of the particular drugs (alkylating agents versus anthracyclins) or perhaps differences in the models used.

Ovarian damage due to chemotherapy might not be restricted to direct toxicity toward the cellular components of ovarian follicles. Follicle loss could also be an indirect consequence of damage to its supporting vasculature. Despite very early reports of gonadal vascular damage from chemotherapy (Nicosia et al., 1985; Marcello et al., 1990), this possible mechanism of
follicle loss has surprisingly received little attention and is often overlooked as a contributor to ovarian dysfunction. In order to support the continual cycles of ovarian follicle development, the ovarian vasculature is constantly remodeling. Primordial and primary follicles rely on passive diffusion from stromal blood vessels, and beyond these stages the growing follicle will develop its own microvasculature. Preantral follicles need to acquire a capillary network for hormonal support and enhanced circulation during antral follicle growth. After ovulation, extensive vascularization of the corpus luteum is essential for the distribution of hormones required for pregnancy. Upon revisiting this type of ovarian damage, it was discovered that ovaries of women previously exposed various types of cytotoxic agents contained blood vessels with thickened walls and narrowed or obliterated lumina (Meirow et al., 2007). Based on evidence of focal fibrosis and the absence of primordial follicles in certain areas within the ovarian cortex, it appeared that this type of vascular damage caused ischemia of cortical tissue areas (Meirow et al., 2007).

The hypothalamic-pituitary-ovarian axis that controls female reproduction is a system which requires cooperation from all endocrine structures involved. Chemotherapy-induced damage to sex steroid-producing follicular granulosa and theca cells or subsequent atresia of antral follicles can lead to ovarian dysfunction that may or may not result in amenorrhea. The following endocrine profiles have been reported in women exposed to chemotherapy: Low inhibin B and E2 levels immediately after chemotherapy, a 10-fold increase in FSH and LH levels after multiple rounds of chemotherapy that returns to normal after 4 months in women that resume menses (Rosendahl et al., 2010), and P4 levels that decline step-wise with each chemotherapy cycle (Mehta et al., 1992). These measurements are all indicative of alteration to the hypothalamic-pituitary-ovarian axis at the level of the ovary. More specifically, they can be explained by damage to the growing follicle population. Chemotherapy has not been shown to alter hormone levels at the hypothalamic or pituitary level, which is a possible effect of cranial or total body irradiation for the treatment of cancer (Chow et al., 2008).

The uterus is another area where chemotherapy and radiotherapy may have dissimilar effects. Myometrial fibrosis, endometrial damage, reduced uterine volume and blood flow have been reported in women (Critchley et al., 1992) and female adolescents (Holm et al., 1999) exposed to abdominal irradiation, especially prepubertally. These complications are associated with unfavourable pregnancy outcomes, such as increased risk of perinatal mortality (Chiarelli et al., 2000). Although vascular damage is clearly evident in the ovary following chemotherapy, there lacks solid evidence of chemotherapy-induced uterine complications (Arnon et al., 2001). However, cyclophosphamide administration has been shown to impair uterine function in mice by reducing plasma E2 from the ovary and also by directly damaging the uterus in a way that alters its response to E2 (Plowchalk et al., 1992). Then again, reports have also claimed that mice have a normal uterine morphological response following chemotherapy administration, but without
indication of the chemotherapy drugs tested or doses used (Critchley et al., 1999). More research is necessary in order to confirm whether or not uterine damage could contribute to the negative impact of chemotherapy on female fertility.

There does not seem to be evidence to support a possible link between congenital anomalies or chromosomal defects and the use of chemotherapy based on studies of children born to chemotherapy-treated childhood cancer survivors (Green et al., 1991; Li et al., 1979). This may be due to the fact that pregnancy prevention is recommended for the following two years post-chemotherapy in case of cancer recurrences. Two years' time rids the ovary of follicles exposed to chemotherapy in the growth phase that might have been particularly vulnerable to germ cell mutation. Using a mouse model to demonstrate pregnancy outcome after follicles were exposed to cyclophosphamide at various stages of development, Meirow and his colleagues discovered that malformation rates were more than 10 times greater following drug exposure. Pups conceived from follicles exposed at an early stage (33%) had the highest chance of malformation, and by the 12th week after treatment, malformation rates returned to normal (Meirow et al., 2001), which demonstrates that DNA damaged oocytes of growing follicles may evade follicle atresia and emphasizes the importance of pregnancy prevention for at least one year after chemotherapy.

**Molecular Mechanisms of Ovarian Damage**

As previously mentioned, the degree of reproductive toxicity due to chemotherapy is largely dependent on the type of drug used. Chemotherapeutic agents are selected based on the best response from the particular type of cancer being treated. Many anti-cancer drugs are cell cycle-specific (CCS), meaning they function by interfering with mitotic cell division, while others are cell cycle non-specific (CCNS) and can target both cycling and quiescent cells. The mechanisms of chemotherapy-induced ovarian damage have been explored for a number of agents in attempt to develop strategies to reduce the risk of premature ovarian failure and infertility in women undergoing treatment. The following sections explain our current knowledge of the molecular mechanisms involved in chemotherapy-induced ovarian damages, summarized by class and in order of decreasing toxicity.

**Alkylating Agents**

The alkylating agent class of chemotherapeutic agents arguably contains some of the most clinically useful drugs for the treatment of cancer today. This group of CCNS drugs includes cyclophosphamide, ifosfamide, busulfan, and carmustine to name a few. Alkylating agents exert cytotoxic effects by transferring their alkyl groups to various cellular constituents, leading to DNA-DNA and DNA-protein crosslinks, and DNA double-strand breaks (DSB). As one of the most
commonly used anti-cancer drugs, and one of the most detrimental to female fertility, cyclophosphamide is the leading drug choice in 'chemo-fertility' studies. Using γ-H2AX as an indicator of DSB, DNA damage has been detected in the oocytes of small follicles shortly after exposure to cyclophosphamide in cultured mouse and rat ovaries (Petrillo et al., 2011). If this DNA damage goes unrepaired, apoptotic pathways are initiated. A number of oocytes and granulosa cells of primordial and growing follicles from mouse ovaries after cyclophosphamide exposure stain positive for terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL), indicating DNA fragmentation in response to apoptotic signaling cascades (Desmeules et al., 2006; Meirow, 2000). Additionally, a mitochondrial-dependent mechanism of apoptotic cell death has been validated by increased levels of pro-apoptotic proteins bax, cytochrome-c and caspase-3 and reduced anti-apoptotic factor Bcl-2 in treated granulosa cells (Zhao et al., 2010).

Together these findings indicate that alkylating agent induced follicle loss may result from intrinsic apoptotic signaling that is initiated by DNA damage.

**Platinum-based Anti-Tumor Drugs**

The three platinum-based analogues cisplatin, carboplatin and oxaliplatin are used to treat a broad range of solid tumors, including ovarian, lung and bladder cancer. They are similar to alkylating agents in that they are CCNS drugs that crosslink DNA. However, they are considered a separate class as they are only ‘alkyl-like’ and do not actually carry an alkyl group. Increased γ-H2AX expression and TUNEL-positive staining have been detected in cisplatin-treated mouse oocytes (Gonfloni et al., 2009). It has been proposed that DNA alkylating drugs stimulate c-abl kinase activity and initiate TAp63-mediated cellular apoptosis in oocytes. Tumor suppressor, TAp63, is a member of the p53 family that is constitutively expressed in oocytes in meiotic arrest and is responsible for maintaining the germline (Suh et al., 2006). Gonfloni and colleagues recently demonstrated that the c-abl-TAp63 pathway is activated following DNA damage caused by cisplatin in human and mouse oocytes. In this pathway, c-abl phosphorylates TAp63 in the presence of DNA double-strand breaks and thereby induces the activation of pro-apoptotic factors to initiate oocyte cell death (Gonfloni et al., 2009).

There is also evidence to suggest that cisplatin can induce cell death by a DNA-damage independent mechanism. Enucleated human melanoma cells express markers of endoplasmic reticulum stress and apoptotic signaling after treatment with cisplatin, while etoposide, a specific DNA-damaging drug (topoisomerase II inhibitor), does not induce apoptotic signaling in cytoplasts. Cisplatin-induced ER stress is likely related to the drug’s non-specific reactivity to various cellular nucleophiles in addition to DNA (Mandic et al., 2003). Additionally, cisplatin administration may cause reduced expression hyperpolarization-activated cation channels on
follicular cells, which has previously been associated with ovarian aging. However, functional significance of these channels with respect ovarian function is still unclear (Yeh et al. 2008).

**Microtubule Inhibitors**

The microtubule inhibitor class of chemotherapy agents includes both taxanes and plant alkaloids. Both subclasses are CCS drugs that specifically target the M-phase of mitosis. The taxane group encompasses paclitaxel and doxetaxel, which inhibit mitosis and stall dividing cells in the G2/M phase of mitosis by preventing microtubule depolymerization (Horwitz, 1994). One study reported that a single dose of paclitaxel (7.5 mg/kg) could reduce primordial follicle numbers in rats to the same extent as cisplatin (5 mg/kg) (Yucebiligin et al., 2004). In contrast, primordial follicle numbers were not found to be significantly reduced in a more recent study in which rats received 5 doses of paclitaxel (5 mg/kg). While they did detect apoptosis in granulosa cells of antral follicles as well as increased follicle atresia and decreased corpora lutea numbers, overall, these effects did not contribute to alterations in endocrine hormone levels, nor were they related to any differences in fertility outcome when the rats were bred 24 days later. For these reasons, the ovarian toxicity of paclitaxel was considered “mild and transient” (Tarumi et al., 2009). Vinblastine, vincristine and vinorelbine are among the plant alkaloid microtubule inhibitors that disrupt microtubule assembly by interfering with tubulin polymerization. Very little is known about the effects of these drugs on female reproduction, as it was reported over a decade ago that there was not a significant risk of ovarian failure associated with their use (Meirow, 2000). However, there is evidence to suggest that plant alkaloids can cause ovarian cell damage. For example, vinblastine injection just prior to superovulation in mice can lead to meiotic arrest and aneuploidy in ovulated oocytes (Russo and Pacchierotti, 1998), a similar effect to what is observed with taxol treatment (Mailhes et al., 1999).

**Antitumor Antibiotics**

DNA-damaging anti-tumor antibiotics are a group of natural anti-cancer drugs produced by Streptomyces bacteria. It includes mitomycin, bleomycin and anthracyclins, such as doxorubicin. The majority of antitumor antibiotics are CCNS, and of this group, doxorubicin is the most widely used. Doxorubicin disrupts DNA replication by intercalating with DNA and by partially inhibiting topoisomerase II (Jurisicova et al, 2006). Consequently, actively dividing cells of the ovary, namely the granulosa cells and endothelial cells, should be particularly susceptible to doxorubicin. This drug, however, was originally considered only mildly toxic to the ovaries. In fact, one study detected a statistically significant increase in pregnancy rates in women that had received doxorubicin treatment in their childhood (Green et al., 2004). This association was considered misleading for a lack of explanation, and with growing evidence of doxorubicin-induced ovarian toxicity, it is now categorized as medium-risk (Blumenfeld, 2012). Granulosa
cells and oocytes in mice that received a single injection of doxorubicin stained positive for TUNEL. Caspase-3 is a key effector in the apoptotic cascade in granulosa cells after doxorubicin administration (Ben-Aharon et al., 2010). In oocytes, bax (Perez et al., 1997) caspase-2 (Jurisicova et al., 2006) and ceramide (Perez et al., 1997) are involved in doxorubicin-induced apoptotic signaling, while p53, an initiator of DNA damage related apoptosis, is not required (Perez et al., 1997).

It is possible that doxorubicin exposure can trigger apoptosis through DNA-independent cellular damage. In cardiomyocytes, doxorubicin causes mitochondrial damage by associating with cardiolipin (Goormaghtigh et al., 1987). This interaction could lead to increased cytochrome-c in the cytosol and initiate apoptosis through caspase activation. There is also evidence to suggest that doxorubicin exposure increases reactive oxygen species (Tan et al., 2010), and thereby initiates apoptosis through endoplasmic reticulum stress. These mechanisms may provide insight to how non-proliferative ovarian cells could be susceptible to doxorubicin.

**Cyclophosphamide**

**Uses**

Cyclophosphamide has been the most widely studied chemotherapy drug with respect to chemotherapy-induced infertility (Petrillo et al., 2006), likely due to its potent ovarian toxicity and frequent use in the treatment of cancers common to young women and children. Cyclophosphamide is a nitrogen mustard belonging to the alkylating agent class. Therefore, it exerts cytotoxic effects via the transfer of an alkyl group to various cellular constituents. Alkylation of DNA, primarily on the N7 position of guanine, produces intrastrand and interstrand DNA crosslinks that can interfere with DNA replication and induce cell death by apoptosis. It is a cell-cycle non-specific (CCNS) agent; therefore, although it mainly targets proliferating cells, resting cells are susceptible to its damaging effects as well. Cells can acquire resistance to cyclophosphamide by augmented DNA damage repair or increased conjugation with cellular glutathione (Gamcsik et al., 1999).

A broad spectrum of human cancers and various autoimmune disorders are currently being treated with cyclophosphamide. It is one of the most frequently used chemotherapy drugs, especially for the treatment of childhood and adolescent malignancies such as leukemia, Hodgkin’s and non-Hodgkin’s lymphoma, neuroblastoma and retinoblastoma (Petrillo et al., 2006; Chematilly et al., 2006). Furthermore, cyclophosphamide is most commonly used to treat other cancers that are diagnosed in reproductive-age women (Petrillo et al., 2006) including, but not limited to breast cancer (Anderson et al., 2006), bone and soft tissue sarcoma (Mulder et al., 2012), and ovarian cancer (Handolias et al., 2013). In addition to its anti-neoplastic action,
cyclophosphamide is known to have potent immunosuppressive effects and is also used to treat chronic autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis (Brodsky, 2002). Very high doses of cyclophosphamide are administered in combination with total body irradiation as a myeloablative conditioning regimen for allogeneic haematopoietic stem cell (HSC) transplants. In this setting, cyclophosphamide has two distinct applications since it can be used to eradicate cancer cells while also preventing rejection of donor HSCs (Luznik et al., 2010; Lee et al., 2013).

**Treatment Doses**

Cyclophosphamide can be used therapeutically over a wide range of doses and treatment schedules, depending on the specific disease and patient being treated. Doses can be as low as 2 mg/kg/day, whereas high doses may exceed 6000 mg/m² body surface area (Genre et al., 2002; de Jonge et al., 2005). Conventional MTD administration of cyclophosphamide is generally administered intravenously at 600-750 mg/m² every 3 weeks (Penel et al., 2012). When used for its immunosuppressive effects, cyclophosphamide is often administered at a dose of 100 to 200 mg per day (de Jonge et al., 2005). It is available for both oral and intravenous administration, making it a good candidate for LDM chemotherapy, by which it currently been used in the range of 25-100 mg per day (Penel et al., 2012).

**Pharmacokinetics**

As an inactive prodrug, cyclophosphamide requires hepatic biotransformation. It is metabolized by microsomal mixed-function oxidases to form 4-hydroxycyclophosphamide (4HC), which is in equilibrium with its tautomer, aldophosphamide. In circulation, these unstable intermediates will spontaneously decompose to acrolein and phosphoramidé mustard; the latter being the principal metabolite responsible for DNA alkylation (Colvin et al., 1976). Cellular uptake of the drug will only readily occur in the form of 4HC, and therefore only intracellularly formed PM contributes to its cytotoxicity (Boyd et al., 1986). The additional product, acrolein, is believed to enhance the damaging effects of PM by molecularly altering the cell, although it is not the major anti-tumor agent. Acrolein will react with cellular nucleophiles and cause altered gene expression, reduced cell proliferation and increased susceptibility to apoptosis (Kehrer et al., 2000). Acrolein may also facilitate cellular damage by reducing cellular glutathione, a molecule that functions to eliminate reactive electrophiles such as 4HC (Lee, 1991; Richard and Siemann, 1995). An overview of the metabolism of cyclophosphamide is summarized in Figure 2.

Systemic concentrations of cyclophosphamide have been measured following a number of different treatment regimens. Plasma 4HC levels more accurately reflect active drug availability; however, the instability of this metabolite makes it difficult to measure. Due to its
reactivity and spontaneous decomposition, the plasma half-life of the 4HC metabolite is only several minutes (Hong et al., 1991), and for this reason, stabilization techniques are required in order for levels to be accurately estimated. Peak plasma levels of 4HC have been detected by half an hour to 4 hours following both intravenous infusion and oral administration of cyclophosphamide (Struck et al., 1987). Cyclophosphamide metabolism and activation do not vary with oral and intravenous routes of administration (Struck et al., 1987), but repeated daily exposure will enhance its metabolism. Autoinduction of microsomal enzymes is detectable within 24 hours of cyclophosphamide administration, and as a result of increased metabolism, continuous exposure will gradually elevate peak plasma levels of 4HC while reducing the elimination half-life. Since the elimination rate is inversely correlated with the rate of formation of 4HC, the plasma half-life of cyclophosphamide will vary depending on dose and infusion time. In general, it will range from 5 to 9 hours with almost complete elimination by 24 hours after treatment for both MTD and LDM regimens (de Jonge et al., 2005). Long-term administration of cyclophosphamide eventually leads to stable levels of active 4HC. Thus, enhanced drug metabolism is not considered a potential mechanism of cyclophosphamide resistance (Emmenegger et al., 2007).

**Figure 2: Metabolism of cyclophosphamide (4-hydroperoxycyclophosphamide; 4HC).** Cyclophosphamide requires activation by microsomal enzymes (P450) in order to exert its cytotoxic effects. In vitro, 4HC is used to bypass the need for activation of the drug. In aqueous solutions, 4HC is converted to 4-hydroxycyclophosphamide. Cellular uptake occurs in the form of 4-hydroxycyclophosphamide, which is further metabolized to phosphoramide mustard and acrolein inside the cell. Phosphoramide mustard is the major toxic metabolite of cyclophosphamide, while acrolein is believed to enhance cell damage. Decomposition products are indicated by the dotted arrows. (Images of chemical structures obtained from de Jonge et al., 2006).
Current Fertility Preservation Options

Over the past decade, there has been a considerable amount of research related to fertility preservation in women undergoing chemotherapy, as the current options are not ideal. Since it is best not to delay treatment, a woman with concerns about fertility should be immediately referred to a specialist. Cryopreservation and ovarian suppression are the only options presently available to women that require chemotherapy.

Cryopreservation

Advances in cryotechnology have allowed embryos, oocytes and ovarian tissue to be frozen for future pregnancy (Donnez et al., 2004). Ideally, these procedures should take place before the initiation of chemotherapy; however, in many cases treatment cannot be postponed (Abir et al., 2008). For a number of women, cryopreservation only becomes an option after they have already undergone at least one round of chemotherapy, which could drastically reduce success rates.

Gonadotropin-Releasing Hormone (GnRH) Analogues

Gonadotropin releasing hormone (GnRH) agonists are currently available for clinical use, but their usefulness for fertility preservation is debatable. Based on the notion that follicles are less vulnerable to the toxic effects of chemotherapy if they are maintained in a non-proliferative state, these drugs are believed to provide a protective effect on the ovary by interfering with the hypothalamic-pituitary-axis in ways that suppress folliculogenesis (Badawy et al., 2009). Gonadotropin-releasing hormone agonists stimulate the release of pituitary gonadotropins, causing an initial spike in FSH and LH. After prolonged exposure, GnRH receptors are downregulated in the pituitary as a result of internalization of GnRH agonist-receptor complexes, preventing further stimulation and the release of the gonadotropin hormones. Gonadotropin-releasing hormone antagonists cause a similar, but more immediate response by preventing endogenous action via receptor competition (Tarlatzis, 2007).

It is still unclear whether this approach will benefit patients long-term. Based on various measurements of reproductive outcome, some authors claim that GnRH analogues are useful for fertility preservation (Del Mastro et al., 2011; Wong et al., 2012; Leonard et al., 2010) in women that receive chemotherapy, while others argue that current evidence is inconclusive (Ismail-Khan et al., 2008; Gerber et al., 2011). The majority of these studies have been based on short-term measurement of reproductive outcome, such as the presence amenorrhea, the recovery of menses, or serum levels of hormonal markers FSH, LH and E2, which are better indicators of growing follicle damage. In addition, they have only been evaluated for up to one year after treatment. It is important to consider that women may not show signs of premature ovarian failure
or early menopause until decades after chemotherapy; therefore, short-term measurement of reproductive outcome as the primary end-point likely overestimate the true efficacy of GnRH analogues. Estimation of ovarian reserve based on inhibin B and AMH levels, which are better indicators of follicle reserve, have not yet demonstrated benefits of their use (Gerber et al., 2011). Long-term follow-up studies on the reproductive outcomes of women co-treated with GnRH analogues are necessary to evaluate their true efficacy in fertility preservation during chemotherapy.

**Proposed Pharmacological Interventions**

A number of different protective treatments have been proposed to shield the ovary from damage during chemotherapy exposure. In recent years, there has been a dispute over the effectiveness of the drug Imatinib, a tyrosine kinase inhibitor suggested for use alongside cisplatin treatment to prevent germ cell damage. It is currently used to treat Philadelphia chromosome-positive chronic myelogenous leukemia (CML), which arises from a chromosomal translocation that produces a hybrid chromosome by fusion of the BCR and ABL genes. The resulting chimeric BCR-ABL gene leads to cancer due to high levels of tyrosine kinase activity. Imatinib targets the abl kinase domain, and promotes the autoinhibition of c-abl in addition to bcr-abl (Nagar et al., 2007). Inhibition of the c-abl-TAp53 pathway by Imatinib has been claimed to protect against oocyte apoptosis and prolong fertility in mice treated with cisplatin (Gonfloni et al., 2009, Maiani et al., 2012). However, there is also evidence in the literature that contradicts the potential ovarian protective effects of imatinib. Kerr and associates have argued that cisplatin-induced oocyte apoptosis and primordial follicle loss are not prevented by pre-treatment with Imatinib, based on testing in two separate mouse strains (Kerr et al., 2012). Additionally, in a clinical case report it was stated that a woman who had been treated for CML with Imatinib had a severely compromised ovarian response after gonadotropin stimulation, but a normal response when taken off Imatinib (Zamah et al., 2011).

The inhibition of apoptotic pathways in ovarian cells during exposure to chemotherapy has also been explored as a possible approach to fertility preservation. Sphingosine-1-phosphate (SIP), an anti-apoptotic metabolite of ceramide, was proposed for use with doxorubicin to inhibit the ceramide-promoted apoptotic pathway in oocytes. Apoptosis is blocked in vitro when oocytes are treated with SIP in combination with doxorubicin (Perez et al., 1997) and in vivo it has been demonstrated to have a protective effect on the primordial follicle pool (Hancke et al., 2007). Similarly, 3,5,3′-triiodothyronine (T3) prevents paclitaxel-induced apoptosis in rat granulosa cells (Verga et al., 2012). While this approach may protect ovarian cells from the cytotoxic effects of chemotherapy drugs, there is still insufficient evidence to confirm that cancer cells do not also benefit. Also, due to alternate mechanisms of apoptotic cell death associated with the various
chemotherapy drugs, different apoptosis-blocking drugs would need to be developed for each drug or drug type. Their usefulness is even further limited by the fact that cancer is rarely treated with just one chemotherapeutic agent and the likelihood that there are multiple ovarian targets of chemotherapy.

**Summary**

It is well established that chemotherapy drugs have harmful effects on ovarian function and the reproductive potential of children, adolescents and premenopausal women, which can negatively impact the quality of life of young female cancer survivors. Various characteristics of the ovary make it particularly vulnerable to the toxic effects of chemotherapy. As a result, there are a number of possible mechanisms by which chemotherapy can deplete the ovarian reserve and cause infertility. Current options for fertility preservation in women with cancer are not ideal. Oftentimes the urgency for chemotherapy does not allow cryopreservation techniques to be performed immediately and long-term benefits of GnRH analogues have not yet been established. This emphasizes the need for better targeted cancer therapies that will have reduced effects on ovarian function and female fertility.
RATIONALE

Females that undergo chemotherapy for the treatment of cancer prior to the natural age of menopause are at risk of treatment-related infertility. This can have a profound negative impact on the quality of life of young cancer survivors, and unfortunately, current fertility preservation options are limited. Due to the urgency of cancer treatment, cryopreservation may not be a viable option until after chemotherapy has already been initiated, at which point these techniques may be unsuccessful. The use of gonadotropin analogues alongside chemotherapy administration is a possible alternative, although the long-term benefits of this approach have not been verified.

Premature ovarian failure or early menopause can result from accelerated depletion of the ovarian reserve, which could be a consequence of direct primordial follicle damage or an indirect response to growing follicle loss. In either case, premature ovarian failure may be more specifically due to follicular cell death or damage to the follicle-supporting vasculature. There is evidence to support each of these possible mechanisms in the literature; however, the type and severity of chemotherapy-induced ovarian damage is dependent on the type of drug used.

In addition to drug type, factors that have been found to influence the risk of long-term ovarian damage include the cumulative dose received and the age of the patient at the time of exposure to chemotherapy. To date, the scheduling of chemotherapy administration has not been identified as an associated risk factor. This study was designed to test the hypothesis that the Low-Dose Metronomic (LDM) chemotherapy administration strategy is less detrimental to ovarian function than the traditional Maximum-Tolerated Dose (MTD) strategy.

Objectives:

1. Evaluate the toxicity of LDM and MTD cyclophosphamide (4HC) schedules on granulosa cells in vitro.

2. Compare the effects of LDM and MTD cyclophosphamide schedules on ovarian morphology and function in vivo.

3. Determine the effects of LDM and MTD cyclophosphamide administration on the ovarian vasculature and the regulation of angiogenesis in vitro and in vivo.
MATERIALS AND METHODS

Cell Culture Model

Treatment Schedules

Spontaneously immortalized rat granulosa cells (SIGCs) were generously provided by Dr. R. Burghardt (Department of Anatomy, Texas A&M University, College Station, TX). They were cultured in T-75 flasks (Fisher Scientific, Whitby, ON) containing Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibco, Life Technologies Inc., Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Life Technologies Inc., Burlington, ON) and 1% antibiotic-antimycotic (ABAM; Life Technologies Inc., Burlington, ON). The growth kinetics of the SIGCs were determined for various seeding densities in 96-well plates, 24-well plates on glass coverslips, and in 10cm dishes. Seeding densities that resulted in cellular confluence of 80% after 144 hours were chosen for treatments in each culture vessel. Cells were treated with LDM or MTD schedules of active metabolite of cyclophosphamide, 4-hydroperoxycyclophosphamide (4HC; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 6 days (144 hours). A 5mg/ml stock solution of 4HC in PBS (Life Technologies Inc., Burlington, ON) was prepared and immediately stored at -20°C in 10μl aliquots. Fresh aliquots were thawed prior to each treatment and used immediately. The general structure of the in vitro treatment schedules are shown in Figure 3.

![Figure 3: Schematic diagram of in vitro 4HC dosing schedules.](image)

Figure 3: Schematic diagram of in vitro 4HC dosing schedules. Spontaneously immortalized rat granulosa cells (SIGCs) were treated with 4HC by schedules representative of either MTD (red arrows) or LDM (blue arrows) chemotherapy. Various time points over the course of the 144 hour treatment schedule were selected for analysis of cellular viability, apoptosis, proliferation, DNA damage, and the expression of vascular factors.

Cellular Viability Assay

A dose-response trial was performed to determine the effect of 4HC scheduling on SIGC viability. Cells were seeded at a density of 200 cells per well in 96-well plates (Fisher Scientific, Whitby, ON). Separate plates were used for each treatment dose to prevent cross-contamination.
of neighboring wells by cytotoxic volatile decomposition products of 4HC (Flowers et al., 2000). Treatments were initiated 24 hours after plating and were carried out for 144 hours total. The LDM treatment groups received doses of 0.1, 1, 10, 100 or 1000 nM 4HC every 24 hours. The MTD schedule consisted of 3000 nM 4HC treatments on days 1 and 4 (T1, T4) and cells were provided with fresh media on the remaining treatment days (T2, T3, T5, and T6). The vehicle control (PBS; Life Technologies Inc., Burlington, ON) group received fresh media daily, supplemented with the same volume of PBS as the highest LDM treatment dose (1000 nM). Each treatment group consisted of 6 technical replicates. Treatment schedules are shown in Figure 3.

Cell viability was measured at 72 hours (prior to treatment) and 144 hours with a water-soluble tetrazolium salt (WST)-1 assay (Roche Applied Science, Laval, QC). Briefly, plates were washed with PBS and incubated with media containing WST-1 (1:10 dilution) and incubated at 37°C. The WST-1 reagent was added to individual plates 1 minute apart to account for the time required to read each plate. Absorbance was measured at 450 nm using a microplate reader after 30 minutes of incubation, and every hour for a total of 4 hours. To control for background differences at each reading time, WST-1 solution was also added to wells on each plate that did not contain cells (blanks; n=3). Cell viability was expressed as a percentage relative to the PBS vehicle control after subtracting the appropriate average ‘blank’ from each absorbance reading.

Immunofluorescence in Formalin-Fixed Cells

The in vitro model of LDM and MTD scheduling on SIGCs previously described (Figure 3) was repeated in 24-well plates (Fischer Scientific, Whitby, ON) on sterile glass coverslips (Micro Cover Glasses, Round; VWR International LLC, Mississauga, ON) for immunofluorescence (n=3/group). The 1000 nM/day LDM schedule was used for comparison with the MTD schedule (3000 nM/3 days) since it resulted in exposure to equivalent cumulative doses at 72 and 144 hour time points. On separate plates for each treatment group, SIGCs were seeded at a density of 1000 cells/well. At 78 and 144 hours, treated cells were fixed with 10% neutral buffered formalin (Fisher Scientific, Whitby, ON) for 1 hour at room temperature and stored in PBS (Appendix II) at 4°C prior to staining by immunofluorescence (IF). Cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Canada Ltd., Oakville, ON) in PBS for 15 minutes at room temperature, washed twice with PBS and blocked in 5% BSA/0.1% sodium azide (Appendix II) for 30 minutes at room temperature. Cells were incubated with appropriate dilutions of primary antibody (Table II) in antibody diluting fluid (Appendix II) overnight at 4°C. The following day, plates were washed with PBS and cells were incubated with the appropriate AlexaFluor conjugated secondary antibody (Table II; Life Technologies Inc., Burlington, ON) for 1 hour at room temperature. Secondary antibodies were rinsed with PBS and cells were incubated in a DAPI solution (0.5μl in 10ml PBS) for 1 minute at room temperature for nuclear counterstaining. The glass coverslips were then rinsed with PBS and mounted on glass slides.
using ProLong Gold Antifade Mountant (Life Technologies Inc., Burlington, ON). The mounting reagent was allowed to cure at room temperature for 24 hours prior to storing the slides at -20°C.

**Image Analysis In Vitro**

Immunofluorescent staining of Cleaved caspase-3 and PhosphoHistone-H3 in SIGCs were imaged using an Olympus BX61 automated upright microscope with MetaMorph Microscopy Automation and Image Analysis software (Molecular Devices, Downingtown, PA). Three fields of view were imaged at 200x magnification for each treatment replicate. Total cell numbers per field of view were determined using CellProfiler 2.0 cell image analysis software (Kamentsky et al., 2011; www.cellprofiler.org), or counted by hand using the "Cell Counter" plugin for ImageJ (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/) for images of lower cellular density (78 hour time points). Immunopositive nuclei were counted by hand and represented as a proportion of total nuclei per field of view. For quantification of γ-H2AX expression, 3 fields of view were imaged at 400x magnification for each treatment replicate. The total number of foci in each cell nucleus was counted by hand using ImageJ software as previously described. The average number of foci per cell nucleus was then determined for each treatment group.

**Western Blot Analysis**

The in vitro model of LDM and MTD scheduling previously described (Figure 3) was repeated in 10cm culture dishes (seeding density: 75,000 cells; n=3/group) using schedules that produced equivalent cumulative doses by the end of the treatment course (LDM:1000 nM/day; MTD: 3000 nM/ 3 days). Protein was extracted from treated cells at 78 and 126 hour time points. At these times, conditioned media samples were also collected and stored at -80°C. Plates were washed with PBS and cells were lysed over ice in a radioimmunoprecipitation assay (RIPA) buffer supplemented with fresh protease inhibitors (Appendix II) for 30 minutes. Buffer samples containing lysate were spun at 21,000 x g for 10 minutes at 4°C. The aqueous phase of each sample was aliquoted and stored at -80°C. Protein concentrations of cell lysate and conditioned media were determined using a DC Bio-Rad Protein Quantification Kit (Bio-Rad, Mississauga, ON).

A wet/tank blotting system (Bio-Rad Mini Trans-Blot Cell; Bio-Rad, Mississauga, ON) was used to prepare western blots. Equal-volume protein samples (20, 40, or 50μg; n=3/group) were prepared in 3x reducing buffer containing dithiothreitol (DTT; Life Technologies Inc., Burlington, ON) denaturing agent (Appendix II) and heated to 90°C for 5 minutes. Denatured samples were loaded in suitable (Table III) separating gels (6% or 12%, Appendix II; 4-15% gradient gels, Bio-Rad, Mississauga, ON) and subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) at 120 volts (V) in room temperature running buffer (Appendix II).
Precision Plus Protein Dual Colour Standards (Bio-Rad, Mississauga, ON) were used as molecular weight markers. When the desired separation was achieved, gels were equilibrated in transfer buffer (Appendix II) for 15 minutes and transferred onto a nitrocellulose membrane (Ammersham Hybond ECL; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) at 100V in ice-cold transfer buffer, for 90 to 120 minutes, depending on the protein of interest (Table III). Following the transfer, membranes were washed in Tris-buffered saline with Tween 20 (TBST; Appendix II) and blocked with either 5% (w/v) BSA or skim milk (SM) in TBST (Appendix II). After blocking for 1 hour, membranes were incubated in the appropriate primary antibody solution (Table III) overnight at 4°C. The following day, membranes were washed with TBST and incubated in the corresponding horseradish-peroxidase (HRP) conjugated secondary antibody solution (Table III) for 1 hour at room temperature. Membranes were washed again in TBST and antibody binding was detected by enhanced chemiluminescence (ECL) (Clarity Western ECL Substrate; Bio-Rad, Mississauga, ON). Signals were detected and imaged using a Bio-Rad ChemiDoc XRS+ system and relative densitometry was determined using Bio-Rad Image Lab software.

Animal Model

Treatment Schedules

Six-week-old C57Bl6 mice (Charles River Laboratories; n=45) were housed at the University of Guelph Central Animal Facility under standard conditions and with free access to food and water. All animal procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Mice were randomly assigned to LDM or MTD cyclophosphamide (Sigma-Aldrich Canada Ltd., Oakville, ON), or phosphate buffered saline (PBS; vehicle control) treatment groups (n=15/group). All treatments were delivered as equal-volume intraperitoneal (IP) injections. The MTD treatments were administered in 6-day cycles, with MTD cyclophosphamide (100 mg/kg body weight) on Day 1 followed by 5 days of PBS injections, for a total of 2.5 cycles. The LDM group received 20 mg/kg bodyweight cyclophosphamide daily, and control mice received daily injections of sterile PBS. Mouse ovarian cycles were synchronized during the final 3 treatment days. On day 13, each mouse was given 2.5 international units (IU) of pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich Canada Ltd., Oakville, ON) IP to stimulate synchronous follicle development and maturation. Ovulation was induced 48 hours post-PMSG (Day 15) by IP injection of 2.5 IU of human chorionic gonadotropin (hCG; Sigma-Aldrich Canada Ltd., Oakville, ON). Ovaries were collected at time points representative of preantral (12 hours post-PMSG), antral (24 hours post-PMSG), ovulatory (6 hours post-hCG), early luteal (12 hours post-hCG) and luteal (24 hours post-hCG) stages of follicle development or corpus luteum formation. Equivalent cumulative doses (300 mg/kg) of
cyclophosphamide were received by the mice by the final day of tissue collection. Treatment schedules and tissue collection time points are summarized in Figure 4.

Figure 4: Schematic diagram of in vivo mouse model. A) The division of mice into one of 15 experimental groups (3 treatment groups x 5 time points each). B) Treatment timeline (in days): mice were treated with LDM (blue arrow) or MTD (red arrow) scheduled chemotherapy with cyclophosphamide for approximately two weeks. Mouse ovarian cycles were synchronized with PMSG followed by hCG 48 hours later (closed circles). Ovarian tissue was collected at time points representative of preantral, antral, ovulatory, early luteal and luteal stages (open circles). Equivalent cumulative doses of cyclophosphamide were administered by the final day of tissue collection (300 mg/kg).

Tissue Collection and Preparation

Mice were humanely euthanized by CO₂ asphyxiation followed by cervical dislocation. Blood was drawn via cardiac puncture and collected in vacutainer serum separator tubes (Becton
Dickson and Company, Franklin Lakes, NJ). Plasma supernatant was collected after blood centrifugation (1200g, 10 minutes), then aliquoted and stored at -80˚C. Ovaries were trimmed of fat and connective tissue and weighed upon collection. One ovary from each mouse was fixed overnight in 10% neutral buffered formalin (Fisher Scientific, Whitby, ON) and transferred to 70% ethanol (ETOH; Greenfield Ethanol Inc., Brampton, ON) at least 24 hours prior to paraffin processing for immunohistochemistry and morphometric analysis. Processed tissues were embedded in paraffin, serial sectioned using a rotary microtome (thickness, 5μm), and mounted on glass slides (Superfrost Plus; Fisher Scientific, Whitby, ON). The remaining ovaries were flash frozen in liquid nitrogen for additional protein analysis and stored at -80˚C.

**Ovarian Follicle Quantification**

Serial sectioned ovarian tissues were subjected to routine haematoxylin and eosin (H&E) staining. Blind counts of follicles and corpora lutea were performed on every 5th section, including sections previously stained for immunohistochemistry. Only follicles with a visible nucleus within the ovarian section being measured were considered for quantification. All visible corpora lutea within a histological section were counted, including those from both current and previous cycles (described in Scudamore, 2013).

Follicles were classified based on definitions from Meyers et al. (2004) with slight modifications. Primordial follicles consisted of an oocyte encapsulated by a single layer of entirely squamous granulosa cells. These follicles were not quantified as their detection using H&E is prone to error (Picut et al., 2008). Follicles were considered activated, and therefore at the primary stage, if a single layer of granulosa cells surrounding the oocyte contained at least 1 cuboidal granulosa cell (Da Silva-Buttkus et al., 2009). Secondary follicles had more than one layer of granulosa cells with no visible antrum. Follicles were classified as early antral if 3 or more granulosa cell layers were present with small areas of follicular fluid. Antral follicles were defined by a single large antral space and in preovulatory follicles the oocyte was surrounded by a clearly defined border of cumulus cells. For graphical interpretation and immunohistochemical analysis, follicle classification was further simplified into general preantral and antral stages, which were defined by the absence or presence of antral space, respectively. Therefore, primary and secondary follicles were grouped as preantral follicles, while early antral, antral, and preovulatory follicles were all considered antral follicles.

Follicles were considered atretic when two or more of the following characteristics could be observed within a single section: a degenerating or absent oocyte, two or more pyknotic granulosa cell nuclei, disorganized granulosa cell layers that appeared to be pulling away from the basement membrane, and folding of the zona pellucida (Yener et al., 2013; Sinanoglu et al., 2013; Byskov, 1978, de Rantones et al., 2006). Zona pellucida remnants (ZPRs) were also
quantified as an indication of late follicle atresia, which appeared as a collapsed eosinophilic band within the ovarian stroma (Meyers et al., 2004; Scudamore, 2013).

Average follicle counts per treatment group were based on the sum of 10 centrally located ovarian sections from each mouse within the group. No correction factors were applied. The total ratio of growing to atretic follicles (excluding ZPRs) throughout follicle development was also calculated for LDM, MTD and PBS treatment groups by combining time points of tissue collection within each group.

**Immunohistochemistry on Paraffin-Embedded Ovarian Sections**

Immunohistochemistry was performed by citrate buffer antigen retrieval. Slides with paraffin-embedded tissue sections were deparaffinized in 3 containers of xylene (Fisher Scientific, Whitby, ON) for 5 minutes each (i.e.3 x 5 minutes), and rehydrated by immersion in decreasing concentrations of ETOH (100%, 90%: 2 x 2 minutes, 70%: 1 x 2 minutes). Following rehydration, the slides were washed in PBS (Appendix II; 1 x 3 minutes) and endogenous peroxidase activity was blocked in by a 10 minute incubation in a 1% solution of hydrogen peroxide (H₂O₂) in PBS (From 30% H₂O₂; Appendix II). A citrate buffer was prepared fresh for antigen retrieval with or without Tween 20 (Table I; Appendix II), and heated to 90°C. Tissue slides were washed in PBS (2 x 3 minutes), immersed in near-boiling citrate buffer for 12 minutes, and remained in buffer upon removal from heat for another 20 minutes to cool. During another set of PBS washes (2 x 3 minutes), tissues were circled with a hydrophobic wax barrier (ImmEdge Hydrophobic Barrier Pen; Vector Laboratories, Burlington, ON). Tissues were blocked for nonspecific antibody binding using a 5% bovine serum albumin (BSA)/0.1% sodium azide blocking solution (Appendix II) for 10 minutes. After blocking, slides were incubated in a solution of antibody diluting fluid (Appendix II) with the appropriate concentration of primary antibody (Table I), overnight at 4°C in a humidity chamber. The next day, tissues were washed in PBS (2 x 3 minutes) and incubated in the corresponding biotinylated secondary antibody solution (Table I) for 2 hours at room temperature. They were then washed in PBS (2 x 3 minutes) and incubated for 1 hour at room temperature in a 1:50 dilution of avidin and biotinylated horseradish peroxidase (ExtrAvidin-Peroxidase; Sigma-Aldrich Canada Ltd., Oakville, ON) in antibody diluting fluid. After washing with PBS (2 x 3 minutes), antibody immunolocalization was detected by incubation with fresh 3, 3'-diaminobenzidine (DAB tablets, 10 mg; Sigma-Aldrich Canada Ltd., Oakville, ON) dissolved in 5mL of reverse osmosis water (RO H₂O). The DAB colour reaction time varied depending on the antibody used (Table I); however, incubation time was kept constant for all treatment groups. Slides were then washed with PBS (2 x 3 minutes) and counterstained with Carazzi's Haematoxylin (Appendix II) until the desired contrast was achieved (20 seconds to 1 minute). After rinsing excess haematoxylin off with water, tissues were dehydrated by increasing
concentrations of ETOH (50%: 2 x 3 dips, 70%: 2 x 30 seconds, 90%: 1 x 1 minute, 100%: 2 x 3 minutes), and then immersed in xylene (2 x 3 minutes). Finally, coverslips were mounted on each slide using Cytoseal-XYL mounting medium (Thermo Fisher Scientific, Nepean, ON).

**Immunofluorescence on Ovarian Cryosections**

Flash frozen ovaries were embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Fisher Scientific, Whitby, ON), cryosectioned (5μm) at -20℃, and transferred to glass slides (Superfrost Plus; Fisher Scientific, Whitby, ON). Cryosections were fixed in acetone, stored in a sealed box at -20℃ and stained within one week. Slides were brought to room temperature prior to staining. They were washed in PBS for 3 minutes and incubated in 5% BSA blocking solution (Appendix II) for 10 minutes. Ovarian cryosections were circled with a hydrophobic wax barrier (ImmEdge Hydrophobic Barrier Pen; Vector Laboratories, Burlington, ON) to hold tissue solutions. Tissues were then incubated in an anti-CD31 antibody solution in antibody diluting fluid (Table II) and stored in a humidity chamber overnight at 4℃. The following day, slides were washed in PBS (2 x 2 minutes) and incubated in the appropriate fluorescent-conjugated secondary antibody solution (Table II) for 1 hour at room temperature in the dark. Without exposing the tissue to light, slides were counterstained in a 4′, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Canada Ltd., Oakville, ON) solution in PBS (0.5 μl in 10 ml) for 1 minute. This solution was rinsed off in PBS (2 x 2 minutes) prior to coverslipping slides using ProLong Gold Antifade Mountant (Life Technologies Inc., Burlington, ON). The mounting reagent was allowed to cure at room temperature for 24 hours. Slides were stored overnight in a light-tight box at -20℃ prior to imaging.

**Image Analysis In Vivo**

Ovarian sections stained by immunohistochemistry were imaged by bright-field microscopy using a Nikon Eclipse E600 microscope with a QImaging camera. Nuclear Ki67 and Cleaved caspase-3 stains were imaged 200x magnification and the percentage of positive nuclear staining were determined using the ImmunoRatio web application (Tuominen et al., 2010; http://153.1.200.58:8080/immunoratio/) or the ImmunoRatio plugin for ImageJ software (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/). The algorithm was calibrated for each immunostain using the “Analysis Settings” under the “Advanced mode” tab for each image capture setting so that the reference image accurately differentiated between DAB and haematoxylin stains. For Ki67, oocytes, which stained positive for Ki67, were removed from the image using ImageJ prior to analysis. Using ImmunoRatio, Cleaved caspase-3 was analyzed based on follicle stage (preantral versus antral) and status (healthy versus atretic) at 600x magnification. These follicles types were also imaged at 600x magnification and analyzed for cytoplasmic vascular factors, VEGF, FLK-1, TSP-1 and CD36. For these factors, the percentage
of positively stained tissue was determined using Aperio ImageScope software (Aperio, Vista, CA). The “Positive Pixel Count v9” algorithm was optimized and used for each immunostain/image setting. Percent positivity was calculated as a proportion of total positive pixels (the sum of weak positive, positive and strong positive pixels). Primary and secondary omission controls for each stain can be found in Appendix III. Example reference markup images for each automated analysis are provided in Appendix IV.

Ovarian cryosections that were fluorescently immunostained for CD31 were imaged at 400x magnification using an Olympus BX61 automated upright microscope with MetaMorph Microscopy Automation and Image Analysis software (Molecular Devices, Downington, PA). Five images were captured per section and image overlays were created by merging separate color channels in ImageJ. Microvessel density was also quantified using ImageJ by tracing CD31 positive blood vessels to obtain the total microvessel area for each field of view. This method was also used to determine the total tissue area so that microvessel density could be represented as a proportion of the ovarian tissue.

Statistics

GraphPad Prism software was used for statistical analysis and graph preparation. Each treatment group was represented by at least 3 replicates in all experiments. Data from the in vitro model was analyzed using a one-way analysis of variance (ANOVA) and a Tukey’s test was used to determine statistical differences among treatment group means. A two-way ANOVA was performed for in vivo data and was also followed by a Tukey’s post-hoc test. Differences among treatment groups were considered significant if \( p < 0.05 \).
Table I: Immunohistochemistry method details.

<table>
<thead>
<tr>
<th>Antigen Retrieval</th>
<th>1° Antibody</th>
<th>Source</th>
<th>1° Solution</th>
<th>2° Solution</th>
<th>DAB time (sec)</th>
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</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>Cl. Casp-3</td>
<td>cs9661</td>
<td>1:300</td>
<td>1:100 (R)</td>
<td>30</td>
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<tr>
<td></td>
<td>VEGF</td>
<td>ab46154</td>
<td>1:400</td>
<td>1:100 (R)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Flk-1</td>
<td>sc6251</td>
<td>1:600</td>
<td>1:100 (M)</td>
<td>30</td>
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<tr>
<td></td>
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<td>1:1000</td>
<td>1:100 (R)</td>
<td>90</td>
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<tr>
<td>Citrate + Tween 20</td>
<td>Ki67</td>
<td>ab15580</td>
<td>1:200</td>
<td>1:100 (R)</td>
<td>30</td>
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<tr>
<td></td>
<td>TSP-1</td>
<td>sc81755</td>
<td>1:50</td>
<td>1:100 (M)</td>
<td>30</td>
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<tr>
<td></td>
<td>CD31</td>
<td>ab28364</td>
<td>1:25</td>
<td>1:100 (R)</td>
<td>60</td>
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</tbody>
</table>

Antibody sources are detailed in full in Appendix I. Antigen retrieval was performed using either Citrate Buffer (Appendix II) or Citrate Buffer supplemented with 0.05% Tween 20 ("Working Citrate with Tween-20 Buffer": Appendix II). Antibody solutions consisted of antibody diluting fluid (Appendix II) with the appropriate antibody dilution ("1°, 2° Solution"). Biotinylated anti-mouse ("M") or anti-rabbit ("R") IgG secondary antibodies (Sigma-Aldrich Canada Ltd., Oakville, ON) were used for secondary solutions.
### Table II: Immunofluorescence method details.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>1˚ Solution</th>
<th>2˚ Solution</th>
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<tr>
<td>In Vitro:</td>
<td></td>
<td></td>
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<tr>
<td>γ-H2AX</td>
<td>ab11174</td>
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<td>1:200 (R)-594</td>
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<td>Cleaved Caspase-3</td>
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<td>1:400</td>
<td>1:100 (R)-488</td>
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<td>PhosphoHistone-H3</td>
<td>ab5176</td>
<td>1:2000</td>
<td>1:100 (R)-594</td>
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<tr>
<td>In Vivo:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td>ab28364</td>
<td>1:25</td>
<td>1:100 (R)-594</td>
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</tbody>
</table>

Antibody sources are detailed in full in Appendix I. Antibody solutions consisted of antibody diluting fluid (Appendix II) with the appropriate antibody dilution (“1˚, 2˚ Solution”). Alexa Fluor 594 or 488-labelled anti-rabbit (“R”) IgG secondary antibodies (Life Technologies Inc. Burlington, ON), were used for secondary solutions.
Table III: Western blot method details.

<table>
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<tr>
<th>1˚ Antibody</th>
<th>Source</th>
<th>% Gel</th>
<th>Protein (μg)</th>
<th>Blocking Solution</th>
<th>1˚ Solution</th>
<th>2˚ Solution</th>
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<td>BSA</td>
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<tr>
<td>Cl. Casp-3</td>
<td>cs9661</td>
<td>12%</td>
<td>50</td>
<td>BSA</td>
<td>1:500-BSA</td>
<td>1:2000 (R)-SM</td>
</tr>
<tr>
<td>VEGF</td>
<td>ab46154</td>
<td>4-15%</td>
<td>20</td>
<td>SM</td>
<td>1:500-SM</td>
<td>1:2000 (R)-SM</td>
</tr>
<tr>
<td>pVEGFR-2</td>
<td>PA1-14441</td>
<td>4-15%</td>
<td>20</td>
<td>BSA</td>
<td>1:500-BSA</td>
<td>1:4000 (R)-SM</td>
</tr>
<tr>
<td>TSP-1</td>
<td>sc81755</td>
<td>6%</td>
<td>20*</td>
<td>SM</td>
<td>1:200-SM</td>
<td>1:2000 (M)-SM</td>
</tr>
<tr>
<td>CD36</td>
<td>NB400-144</td>
<td>4-15%</td>
<td>20</td>
<td>SM</td>
<td>1:2000-SM</td>
<td>1:3000 (R)-SM</td>
</tr>
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<td>Bcl-2</td>
<td>cs2876</td>
<td>4-15%</td>
<td>20</td>
<td>SM</td>
<td>1:1000-SM</td>
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</table>

**Loading Controls:**

<table>
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<th>Source</th>
<th>Dilution</th>
<th>Solution 1</th>
<th>Solution 2</th>
</tr>
</thead>
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<tr>
<td>β-Actin</td>
<td>cs4967</td>
<td>1:5000</td>
<td>SM</td>
<td>1:5000 (R)-SM</td>
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<td>GAPDH</td>
<td>cs2118</td>
<td>1:1000-BSA</td>
<td>1:2000 (R)-BSA</td>
<td></td>
</tr>
<tr>
<td>Amido Black</td>
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</table>

Antibody sources are detailed in full in Appendix I. Antibody and blocking solutions consisted of either 5% bovine serum albumin ("BSA") or 5% skim milk ("SM") in TBST (Appendix II) with the appropriate antibody dilution ("1˚, 2˚ Solution"). Anti-Mouse ("M") or anti-Rabbit ("R") IgG, HRP-linked secondary antibodies (Cell Signalling Technology, Danvers, MA) were used for secondary solutions. Either β-actin or GAPDH was used as the loading control for each blot, or total protein was revealed using an Amido Black solution.

*protein obtained from conditioned media used rather than cell lysate.*
RESULTS

The effects of chemotherapy scheduling on granulosa cell viability, proliferation and apoptosis in vitro

Spontaneously immortalized rat granulosa cells (SIGCs) were exposed to media containing 4HC by schedules representative of LDM or MTD chemotherapy, and cell viability was measured at 72 (midpoint) and 144 hours (endpoint) using a WST-1 assay. After 72 hours, LDM administration of 4HC caused a reduction in SIGC viability compared to the PBS vehicle control group (p<0.05). In comparison, MTD treated cells were significantly less viable than both the PBS and LDM treatment groups (p<0.0001; Figure 5A). By 144 hours, the highest LDM treatment (1000 nM per day) had further reduced cell viability by greater than 50% (p<0.0001); however, the mean reduction in cell viability was still significantly less than that of the MTD treated cell populations (p<0.01; Figure 5B), which consisted of very few viable cells by the end of treatments. Detectable differences in cell viability were not observed at either time point for LDM doses less than 1000 nM per day.

The cumulative dose-equivalent LDM and MTD schedules that significantly decreased SIGC viability (LDM: 1000 nM/day; MTD: 3000 nM/3 days) were examined in subsequent in vitro experiments. Reduced cell viability due to chemotherapy exposure could be explained by decreased cell proliferation, increased apoptotic cell death, or a combination of these effects. Therefore, levels of cell proliferation and apoptosis were evaluated in SIGC populations using immunofluorescence (IF) and Western blot (WB) analyses at various time points throughout treatment. Cell proliferation was measured by PhosphoHistone-H3 immunofluorescence after 78 and 144 hours of treatment. The LDM treatment regimen did not significantly alter cell proliferation at either time point. However MTD treated cell populations were less proliferative than the controls at 78 hours (p<0.001; Figure 6A). Apoptosis was measured by Cleaved caspase-3 immunofluorescence and Western blot analyses at treatment endpoints of 144 and 126 hours, respectively (Figure 7). At 126 hours, Cleaved caspase-3 expression was significantly elevated in both LDM (p<0.05) and MTD (p<0.01) treatment groups relative to the PBS control cultures (Figure 7A, B). Quantification of Cleaved caspase-3 nuclear-positive staining by immunofluorescence at 144 hours revealed an increase in apoptotic cells after MTD treatment relative to PBS (p<0.01) and LDM (p<0.05) treatments (Figure 7C, D), but levels of apoptosis were similar among LDM and PBS control groups. In addition, altered nuclear morphology could be observed in photomicrographs of chemotherapy treated cells fixed and stained for immunofluorescence, indicated by the DAPI nuclear stain (Figures 6C, 7D).
Figure 5: Granulosa cell viability after LDM and MTD treatment schedules of 4HC. A WST-1 assay was used to measure SIGC viability after 72 (A) and 144 (B) hours of 4HC treatments. Cell viability is presented as a percentage of PBS controls. At 72 and 144 hours, equivalent cumulative doses (3000 nM at 72 hours/6000 nM at 144 hours) administered by LDM and MTD schedules (LDM: 1000 nM/day; MTD: 3000 nM/3 days) had both significantly reduced SIGC viability compared to PBS control cultures. At both time points, cells treated by LDM scheduling were still significantly more viable than the comparable MTD treatment. No other LDM treatment doses caused a significant change in cell viability. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparison test. Asterisks directly above bars and lines represent statistical significance relative to PBS and LDM treatment, respectively (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Error bars represent standard error (n=6/treatment group).
Figure 6: Granulosa cell proliferation after LDM and MTD treatment schedules of 4HC.
SIGC proliferation was determined by PhosphoHistone-H3 immunofluorescence after 78 and 144 hours of LDM and MTD 4HC treatments. A) A significant (*p<0.05) reduction in the percentage of proliferating cells was measured after 78 hours of MTD 4HC scheduling relative to PBS and LDM treatments. B) After 144 hours, mitotic rates decreased and there was no longer a significant difference in the proportion of proliferating cells among treatment groups. C) Representative images of PhosphoHistone-H3 (green) expression overlaying a DAPI nuclear counterstain (blue) are shown. Three fields of view imaged at 400x magnification were quantified per treatment replicate (n=3/treatment group). Asterisks directly above bars and lines represent statistical significance compared to PBS and LDM treatment, respectively. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error.
Figure 7: Granulosa cell apoptosis after LDM and MTD treatments of 4HC. Expression of Cleaved caspase-3 was measured by Western blot (WB) and immunofluorescence (IF) to detect apoptosis in response to 4HC exposure. A, B) Immunoblot and densitometric analysis of Cleaved caspase-3 after 126 hour treatments. Expression levels were normalized to GAPDH bands. Cleaved caspase-3 was significantly increased by LDM (*p<0.05) and MTD (**p<0.01) schedules. C) The proportion of positively stained cells was also significantly higher in MTD treated cultures relative to PBS (**p<0.001) and LDM treated (*p<0.05) cultures when quantified by immunofluorescence after 144 hours. D) Representative images of Cleaved caspase-3 (red) expression overlaying a DAPI nuclear counterstain (blue). Three fields of view imaged at 400x magnification were quantified per treatment replicate (n=3/treatment group). Asterisks directly above bars and lines represent statistical significance compared to PBS and LDM treatment, respectively. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error.
Levels of DNA damage induced by LDM and MTD 4HC schedules in vitro

The extent of DNA damage in granulosa cells caused by LDM and MTD schedules was also determined based on γ-H2AX expression levels during and upon completion of chemotherapy treatments. Western blot densitometric analyses were used to measure γ-H2AX levels in SIGCs after 78 and 126 hours. Two bands were detected at approximately 17 and 25 kilodaltons (kDa), corresponding to γ-H2AX and ubiquitinated γ-H2AX, respectively. These bands were considered together as total γ-H2AX expression. Immunofluorescence of γ-H2AX was used to quantify double stranded DNA breaks after 144 hours of 4HC exposure, which could be visualized by positively stained nuclear foci (Figure 8).

Western blot analysis of γ-H2AX revealed higher levels of DNA damage in MTD treated SIGC populations relative to PBS (p<0.001) and LDM treated cells (p<0.01) after 126 hours of treatment, although differences were not detected at the earlier 78 hour time point. The LDM treated cells did not have significantly elevated γ-H2AX levels at either of these time points (Figures 8A-D). Both LDM and MTD treatments induced γ-H2AX nuclear foci in SIGCs 144 hours after the initiation of treatments (p<0.01 and p<0.0001, respectively). Gamma-H2AX expression was very low in PBS treated cell populations (on average, <1 foci per nucleus). In contrast, cells that received LDM and MTD scheduling of 4HC exhibited a 7 and 11-fold increase in the average number of nuclear γ-H2AX foci, respectively (Figure 8E). At this endpoint, the mean number of γ-H2AX foci per cell were statistically similar among the chemotherapy groups, although for the LDM treatment group the mean was represented by few cells with a high number (>20) of nuclear foci, whereas the majority of MTD treated cells had positive γ-H2AX staining, but a relatively lower number of total foci (<10) per nucleus. Abnormal nuclear morphology was also a common characteristic of LDM, but more so MTD treated SIGCs (Figure 8F).
Figure 8: Granulosa cell DNA damage caused by LDM and MTD treatment schedules of 4HC. Expression of γ-H2AX was measured by Western blot (WB) and immunofluorescence (IF) to detect DNA damage from 4HC exposures. A, B) Immunoblot and densitometric analysis of γ-H2AX after 78 hour treatments. Both ubiquitinated (ub-H2AX) and non-ubiquitinated (γ-H2AX) γ-H2AX proteins were included in densitometry measurements. Expression levels were normalized to β-actin bands. Total levels of γ-H2AX did not vary among treatment groups. C, D) Immunoblot and densitometric analysis of γ-H2AX expression after 126 hours of treatment. Gamma-H2AX expression was significantly elevated by MTD scheduling relative to PBS (***p<0.001) and LDM (** p<0.01) treatments. E) When quantified by immunofluorescence at 144 hours, the average number of nuclear γ-H2AX foci per cell was significantly higher in MTD (****p<0.0001) and LDM (**p<0.01) treated cultures relative to PBS controls. F) Representative images of γ-H2AX (green) expression overlaying a DAPI nuclear counterstain (monochrome). Three fields of view imaged at 400x magnification were quantified per treatment replicate (n=3/treatment group). Insets show cell nuclei for better visualization of foci (400x magnification, enlarged image). Asterisks directly above bars and lines represent statistical significance compared to PBS and LDM treatment, respectively. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error.
The effects of chemotherapy scheduling on granulosa cell protein expression in vitro

Western blot analysis was used to evaluate SIGC expression of vascular factors TSP-1, VEGF, CD36 and pVEGFR-2, as well as survival factor, Bcl-2, in SIGCs after 144 hours of in vitro LDM and MTD 4HC schedules. Levels of TSP-1 in conditioned media did not vary among treatment groups (Figures 9A, B), nor did cellular expression levels of VEGF (Figures 9D, E). The TSP-1 receptor, CD36, and phosphorylated VEGF receptor, pVEGFR-2, both appeared to be down-regulated in MTD treated cells (Figures 9C, F). There was also a reduction in Bcl-2 expression in MTD treated cell populations that was not statistically significant (Figures 9G, H).

Figure 9: Granulosa cell expression of vascular and survival factors following LDM and MTD treatment schedules of 4HC. Expression levels of pro- and anti-angiogenic factors VEGF and TSP-1, their respective receptors, pVEGFR-2 and CD36, and cell survival factor, Bcl-2, were measured by Western blot analysis after 126 hours of treatments. A, B) Immunoblot and densitometric analysis of TSP-1 from conditioned media. C) Immunoblot of TSP-1 receptor, CD36. D, E) Immunoblot and densitometric analysis of VEGF. F) Immunoblot of activated VEGF receptor, pVEGFR-2. G, H) Immunoblot and densitometric analysis of Bcl-2. Respective loading controls are displayed in the lower box of immunoblot images for each factor. Differences among each of these factors were not significantly different at this time point (p<0.05). Significance was determined by one-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error (n=3/treatment group).
The effects of chemotherapy scheduling on ovarian morphology and function

Both ovaries from each mouse were trimmed of fat and weighed upon tissue collection. The mean ovarian weights were not significantly different among treatment groups at any stage of ovarian follicle development (Figure 10). However, follicle counts revealed that there were notable differences in the quantity and type of follicles present at comparative time points of tissue collection (Figures 11A, B). At the preantral stage, there was a significant reduction in the total number of preantral follicles in ovaries of LDM treated mice compared to controls (p<0.05) and even fewer preantral follicles were observed in the ovaries of mice that received MTD treatments compared to PBS control mice (p<0.001). At both the preantral and antral stages, there was less evidence of follicle atresia in LDM treated mice relative to PBS (p<0.01) and MTD (p<0.001) treated mice. By the antral stage, a significantly higher number of preantral follicles were found in ovaries of MTD mice compared to LDM mouse ovaries (p<0.05), while there were still significantly fewer preantral follicles in LDM mice relative to the controls (p<0.001). At the ovulatory stage, follicle counts were statistically similar among treatment groups. A second reduction in preantral follicles occurred in the MTD treatment group relative to the controls (p<0.1) and follicle atresia declined in this group relative to LDM treated mice (p<0.001) by the early luteal stage. By the final luteal stage, follicle atresia was significantly higher in MTD treated mice relative to the controls (p<0.01), but the number of each follicle type was statistically similar among treatment groups. Antral follicle numbers in ovaries of each treatment group were not significantly different at any time point.

Although preantral follicle numbers were reduced by LDM treatment, a similar trend was observed in preantral follicle numbers at each stage with respect to the other stages in PBS and LDM treatment groups (Figure 11B). A distinctly different wave of follicle development was detected in MTD treated mice. After the initial measurement reduction at the preantral stage, preantral increased during the antral to ovulatory stages and declined yet again from the ovulatory to early luteal stage of follicle development. This trend was almost opposite to the fluctuations in preantral follicle numbers in PBS and LDM treated mice throughout the ovarian cycle. Discrete differences in ovarian morphology at the ovulatory stage can be observed in Figure 11C. While some follicles reached the preovulatory stage in MTD treated mice, the majority of the growing follicle population was preantral follicles.

Throughout the course of follicle and luteal development, the total ratio of growing to early atretic follicles was similar among LDM and PBS treatment groups. With respect to these groups, MTD scheduling of cyclophosphamide caused a significant reduction in the ratio of growing to early atretic follicles (p<0.01; Figure 12A). However, the total corpora lutea numbers were not significantly altered by either cyclophosphamide treatment (Figure 12B).
Figure 10: Ovarian weights at each stage of follicle development after LDM and MTD treatment schedules of cyclophosphamide. Both ovaries from each mouse were trimmed of fat and connective tissue and weighed upon collection. Differences in ovarian weights at each time point of tissue collection representative of preantral, antral, ovulatory, early luteal and luteal stages of follicle development were not statistically significant. Significance was evaluated by two-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error (n=6/treatment group).
Figure 11A: Ovarian follicle counts. See description on next page.
Figure 11: Ovarian follicle counts after LDM and MTD scheduling of cyclophosphamide in mice. Ovarian follicle counts were performed on ovarian histological sections from LDM, MTD and PBS control treatment groups. Totals are represented as the sum of every 5th centrally located section, for a total of 10 sections. Only follicles with a visible oocyte within these representative sections were quantified. A) Follicle totals at each stage of development. Both early and late stage (ZPR) atretic follicles are included in the “total atretic” group. Asterisks directly above bars and lines represent statistical significance compared to PBS and LDM treatment, respectively. B) Total preantral and antral follicles over time for each treatment group. A smaller population of growing follicles in LDM treated ovaries exhibited similar growth patterns to PBS treated controls; however, follicle dynamics of MTD treated mice did not reflect that of PBS controls. Significance is indicated in the previous figure (A). C) Representative images of ovarian sections from the ovulatory stage of follicle development. Drastic differences in ovarian morphology are visable in the MTD treatment group relative to PBS and LDM groups. Images were captured at 20x magnification. Significance was determined by two-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error (n=3 mice/treatment group).
Figure 12: Ovarian morphology after LDM and MTD scheduling of cyclophosphamide in mice. A) Breakdown of growing follicle population by preantral and antral follicle status (healthy versus atretic) in each treatment group. B) The total ratios of healthy growing to early atretic follicles over the measured course of follicle development. There was a significantly reduced fraction of healthy growing to early atretic follicles in ovaries of mice that received MTD scheduling compared to PBS controls and mice receiving LDM scheduling. C) The total number of corpora lutea was not significantly different following LDM or MTD treatments relative to controls. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error (n=15/treatment group).
The effects of chemotherapy scheduling on ovarian cell proliferation and apoptosis in vivo

Immunohistochemical detection of follicular Cleaved caspase-3 was evaluated at each stage of follicle development as an indicator of cellular apoptosis. Apoptosis was primarily detected in granulosa cells of growing follicles. Cleaved caspase-3 levels gradually increased during the measured ovarian cycle in mice that did not receive chemotherapy. At the preantral stage, follicular Cleaved caspase-3 expression was significantly higher in MTD treated mice compared to those that were administered either PBS (p<0.0001) or LDM (p<0.001) treatments of cyclophosphamide. At the ovulatory stage, staining revealed a second elevation in granulosa cell apoptosis in MTD treated mice compared to controls (p<0.01) and LDM treated mice (p<0.05). There was a small, but non-significant, increase in Cleaved caspase-3 in follicles exposed to LDM chemotherapy at each stage of follicle development relative to the controls (Figure 13).

Detection of Ki67 by immunohistochemistry was used to evaluate the effects of chemotherapy schedules on ovarian cell proliferation. Declining Ki67 levels were measured as follicle development progressed in control mice. Relative to the PBS control group, there was a significant increase in the number of Ki67 positive nuclei in mouse ovarian sections at the ovulatory stage after MTD chemotherapy exposure, and at the early luteal stage after LDM chemotherapy exposure (p<0.05). In all other stages of follicle development, differences in Ki67 expression were not statistically significant (Figure 14).
Figure 13: Follicular cell apoptosis after LDM and MTD scheduling of cyclophosphamide in mice. Ovaries were immunostained for Cleaved caspase-3 to detect apoptosis within follicles. Areas with the highest proportion of positively stained nuclei were imaged at 600x magnification. Three images were captured per mouse ovarian section (n=9 images analyzed/treatment group). There was a significant increase in the percentage of immunopositive cells in follicles of MTD treated mice relative to PBS (****p<0.0001) and LDM treated mice (***p<0.001) at the time point representing preantral follicle development. Cleaved caspase-3 expression was also elevated at ovulatory stage relative to PBS (**p<0.01) and LDM treated mice (*p<0.05). Significance was determined by two-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error.
Figure 14: Ovarian cell proliferation after LDM and MTD scheduling of cyclophosphamide in mice. Ovaries were immunostained for Ki67 as a marker of cellular proliferation. Areas containing both luteal and follicular structures were imaged at 200x magnification. Three images were captured per mouse ovarian section (n=9 images analyzed/treatment group). A significant increase in the proportion of proliferating cells was detected in the MTD and LDM treatment groups at the time points representing the ovulatory and early luteal stages of development, respectively (*p<0.05). Significance was determined by two-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error.
Vascular effects of LDM and MTD chemotherapy schedules

The effects of chemotherapy scheduling on the ovarian vasculature were evaluated by measurements of blood vessel density and follicular expression of vascular factors in mice. Ovarian cryosections were fluorescently stained for CD31 as an endothelial cell marker to allow quantification of stromal blood vessel density at the preantral follicle development time point. Mean blood vessel density was lower in sections of mouse ovaries that had been exposed to MTD scheduling of cyclophosphamide compared to controls and LDM treated mice, but this difference was not considered statistically significant (Figure 15).

Expression of vascular factors TSP-1 and VEGF, and their receptors, CD36 and FLK-1, were evaluated by immunohistochemical staining of the mouse ovarian tissue. Trends in TSP-1 levels in healthy preantral and atretic follicles displayed an inverse relationship to the number of preantral follicles at each time point for all three treatment groups (Figures 16A, 11A). Follicular TSP-1 expression was not different from controls after LDM scheduling of cyclophosphamide, but there were significant changes in TSP-1 expression in preantral, antral and atretic follicles exposed to MTD scheduling (Figure 16A). Expression of VEGF positively reflected follicle numbers at each stage (Figure 17A, 11A) and was an increase in healthy preantral and atretic follicles at the antral stage in MTD treated mice relative to LDM (p<0.05) and PBS (p<0.01) treated mice, respectively. At the ovulatory time point, heightened VEGF levels were detected in both chemotherapy groups (LDM: p<0.01, MTD: p<0.05; Figure 17A). Expression of CD36 was drastically increased by MTD treatment relative to both PBS and LDM mice at both the preantral and ovulatory stages (Figure 18A). There were also altered FLK-1 levels at the antral stage in all follicle types of MTD treated mice (Figure 19A).
Figure 15: Ovarian blood vessel density after LDM and MTD scheduling of cyclophosphamide in mice. Cryosections of ovarian tissue were stained for CD31 by immunofluorescence at the preantral stage follicle development. Five images were captured per mouse ovarian cryosection (n=15 images analyzed/treatment group) at 400x magnification. Blood vessels are pseudocoloured red, and are overlaying a blue DAPI nuclear stain. Blood vessel density is expressed as vessel area over the total tissue area. There was a non-significant reduction in mean blood vessel density in ovaries exposed to MTD scheduling. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparison test. Error bars represent standard error.
**Figure 16: Expression of TSP-1 based on follicle type and status after LDM and MTD scheduling of cyclophosphamide in mice.** Ovaries from each treatment group were immunostained for anti-angiogenic factor, TSP-1. Preantral, antral, and atretic follicles were imaged at 600x magnification. Three images were captured per follicle type in each mouse unless there were insufficient follicle numbers within the immunostained section. A) Graphical representation of TSP-1 levels at each time point with respect to follicle type. B) Representative images of healthy preantral follicles from the early luteal time point when expression of TSP-1 was significantly higher in follicles exposed to MTD treatment relative to PBS and LDM treatments. Significance was determined by two-way ANOVA followed by Tukey’s multiple comparison test where n≥3 follicles per treatment group. Error bars represent standard error. (*significant to PBS; #significant to LDM).
Figure 17: Expression of VEGF based on follicle type and status after LDM and MTD scheduling of cyclophosphamide in mice. Ovaries from each treatment group were immunostained for pro-angiogenic factor, VEGF. Preantral, antral, and atretic follicles were imaged at 600x magnification. Three images were captured per follicle type in each mouse unless there were insufficient follicle numbers within the immunostained section. A) Graphical representation of VEGF levels at each time point with respect to follicle type. B) Representative images of atretic follicles from the antral stage when expression of VEGF was significantly higher in follicles exposed to MTD treatment relative to PBS treatment. Significance was determined by two-way ANOVA followed by Tukey’s multiple comparison test where n≥3 follicles per treatment group. Error bars represent standard error. (*significant to PBS; #significant to LDM).
Figure 18: Expression of CD36 based on follicle type and status after LDM and MTD scheduling of cyclophosphamide in mice. Ovaries from each treatment group were immunostained for TSP-1 receptor, CD36. Preantral, antral, and atretic follicles were imaged at 600x magnification. Three images were captured per follicle type in each mouse unless there were insufficient follicle numbers within the immunostained section. A) Graphical representation of CD36 levels at each time point with respect to follicle type. B) Representative images of atretic follicles from the ovulatory stage when expression of CD36 was significantly higher in follicles exposed to MTD treatment relative to both PBS and LDM treatments. Significance was determined by two-way ANOVA followed by Tukey’s multiple comparison test where n≥3 follicles per treatment group. Error bars represent standard error. (*significant to controls; **significant to LDM).
Figure 19: Expression of FLK-1 based on follicle type and status after LDM and MTD scheduling of cyclophosphamide in mice. Ovaries from each treatment group were immunostained for VEGF receptor, FLK-1. Preantral, antral, and atretic follicles were imaged at 600x magnification. Three images were captured per follicle type in each mouse unless there were insufficient follicle numbers within the immunostained section. A) Graphical representation of FLK-1 levels at each time point with respect to follicle type. B) Representative images of healthy antral follicles from the antral stage when expression of FLK-1 was significantly lower in follicles exposed to MTD treatment relative to LDM treatment. Significance was determined by two-way ANOVA followed by Tukey’s multiple comparison test where n≥3 follicles per treatment group. Error bars represent standard error. (*significant to controls; #significant to LDM).
DISCUSSION

This study has identified chemotherapy scheduling as a novel risk factor associated with chemotherapy-induced ovarian damage in reproductive age women. We have demonstrated that MTD chemotherapy has more detrimental short-term effects on ovarian function than LDM scheduling. Higher levels of DNA damage and apoptosis were detected in ovarian cells exposed to MTD cyclophosphamide compared to equivalent doses administered in an LDM fashion. In addition, both increased follicle activation and atresia were observed following MTD, but not LDM, administration of chemotherapy in vivo.

There is a clear association between the use of chemotherapeutics and early menopause or infertility as a result of long-term ovarian damage (Letourneau et al., 2012). Factors that contribute degree of ovarian damage caused by chemotherapy drugs include: the drug dose, the number of treatment cycles endured, age at treatment and the type of chemotherapeutic agent used. Alkylating agents have been identified as the most damaging class of chemotherapeutic drugs to female fertility. Of this class, cyclophosphamide has been the most commonly studied for its harmful effects on female fertility (Petrillo et al., 2006). This is likely due to its frequent use for the treatment of cancer in young females. Since the initial application of chemotherapeutics in cancer, maximum-tolerated dose (MTD) drug scheduling has been thought to be the most beneficial approach with respect to tumor cell kill despite its associated toxicity. Recently, however, it has been repeatedly demonstrated that when current chemotherapy drugs are administered at frequent low doses they exert enhanced anti-angiogenic action, as well as other anti-tumor effects (Browder et al., 2000; Klement et al., 2000). This scheduling approach is now commonly referred to as low-dose metronomic (LDM) chemotherapy. As a widely useful and relatively inexpensive chemotherapeutic that can be delivered orally, it is not surprising that cyclophosphamide is the most commonly used drug for LDM scheduling (Lien et al., 2013). Although LDM chemotherapy is associated with little to no short-term side effects, the long-term effects of this scheduling approach are currently unknown.

We compared the effects of LDM and MTD chemotherapy on schedules on ovarian function, which can lead to permanent infertility. These effects were evaluated by exposing ovarian cells and tissue to equivalent cumulative doses of cyclophosphamide by either scheduling approach while holding all other currently known risk factors constant. Chemotherapy schedules with cyclophosphamide were modeled in vitro on isolated rat granulosa cells (SIGCs), and in vivo using 6-week old, sexually mature, female C57Bl/6 mice.
Chemotherapy Dosing Schedules

Six-day treatments on cultured rat granulosa cells

Doses for in vitro analyses were determined using a WST-1 viability assay. Cyclophosphamide is a prodrug that requires activation by liver enzymes. Therefore, active metabolites of cyclophosphamide must be used in vitro in order to examine its toxicity. The activity of cyclophosphamide can be evaluated in vitro using 4-hydroperoxycyclophosphamide (4HC). In aqueous solutions, 4HC is spontaneously reduced to 4-hydroxycyclophosphamide and is then converted to phosphoramide mustard, the active alkylating metabolite. Previously, extended LDM exposure to 4HC in vitro was found to reduce proliferation rates of human endothelial cells (human umbilical vein endothelial cells; HUVECs), but not breast cancer cells (MDA-MB-435), at concentrations ranging from 0.1 to 100 nM (Bocci et al., 2002). Therefore, these doses were considered to be the anti-angiogenic window of 4HC. We used a similar 6-day in vitro model to evaluate granulosa cell sensitivity to LDM and MTD scheduling of 4HC. Cultured SIGCs were continuously exposed to a range of low doses (0.1-1000 nM) of 4HC by refreshing the media and drug solution every 24 hours to represent LDM scheduling. A treatment group to represent MTD chemotherapy was also included, which received a high dose of 4HC once every 3 days. Fresh treatment-free media was supplied to the cells 24 and 48 hours after MTD 4HC treatments to represent rest period of two successive, 3-day cycles.

We did not see a significant reduction in SIGC viability after 144 hour LDM 4HC exposure at concentrations previously determined to be anti-angiogenic (Bocci et al., 2002). The dose response of SIGCs to LDM 4HC concentrations was strikingly similar to one previously reported for the MDA-MB-435 breast cancer cell line, as both cell types were sensitive to 1000 nM 4HC, but not LDM treatments of 100 nM or less (Bocci et al., 2002).

The SIGCs were more susceptible to the effects of MTD treatment than the equivalent LDM treatment with 4HC. We measured a significant decline in SIGC viability at both the treatment midpoint (72 hours; 3000 nM total) and endpoint (144 hours; 6000 nM total) in wells that received 1000 nM per day (LDM) and 3000 nM every 3 days (MTD). Despite the time points being 24 and 72 hours from the last LDM and MTD treatment, respectively, granulosa were significantly more viable if they received LDM 4HC. This supported our hypothesis that frequent low dose schedules are less cytotoxic to ovarian cells than schedules involving intermittent high doses. Even though the LDM and MTD treatment doses that reduced SIGC viability both technically fell within the range of achievable 4HC serum concentrations following administration of normal to high dose cyclophosphamide in humans (Emmenegger et al., 2007), these effective treatment doses were chosen for further analysis based on the observation of a clear schedule-dependent treatment effect on granulosa cell viability. Our ‘LDM’ 4HC dose is still associated with
enhanced anti-angiogenic effects since it causes a greater reduction in the proliferation of HUVECs than MDA-MB-435 cells, whereas the breast cancer cell line is more susceptible when the dose is further increased (Bocci et al. 2002). Though, clinically relevant metronomic concentrations of 4HC would be substantially lower than our selected dose used to represent LDM chemotherapy on cultured SIGCs, but we had already demonstrated that the viability of these cells was not altered by treatment doses within the ‘anti-angiogenic window’ of 4HC in vitro.

Two-week chemotherapy schedules in female mice

We used an in vivo mouse model to replicate ovarian exposure to chemotherapy during LDM and MTD treatment schedules of cyclophosphamide. Although the murine estrous cycle is only 4 to 5 days in duration, it is in many ways similar to the 28 day human menstrual cycle (Caligioni, 2009). Therefore, mice are useful models to evaluate the effects of chemotherapy on the ovary. Metronomic dosing of chemotherapy drugs in humans is still largely empirical due to the lack of validated surrogate markers to measure the anti-angiogenic effects of treatments. Most human clinical trials involving LDM cyclophosphamide currently utilize a fixed oral metronomic dose of 50 mg per day (Emmenegger et al., 2007). In comparison, conventional MTD cyclophosphamide in humans is typically administered in cycles consisting of a single dose 600 to 750 mg/m², once every 3 weeks (Penel et al., 2012). That would make the daily metronomic dose of cyclophosphamide less than a 5% of the MTD; however, the cumulative dose received during a 3-week period is roughly the same (LDM: 1050 mg; MTD: 960-1200 mg for an average body surface area of 1.6m²).

Similar drug proportions are used for LDM and MTD chemotherapy in mouse cancer treatment models. In the literature, metronomic schedules of cyclophosphamide in mice generally range from 10 to 25 mg/kg per day (Penel et al., 2012). Shaked and colleagues consider the optimal biologic dose of metronomic cyclophosphamide to be 20 mg/kg when delivered daily, based on treatment response in 4 different preclinical tumor models. This dose was also associated with the greatest reduction in viable VEGFR-2 positive CEPs in all models, and thus suggested that anti-angiogenic activity of the drug was contributing to enhanced tumor reduction (Shaked et al., 2005). Alternatively, MTD schedules in mice commonly consist of 105 to 150 mg/kg treatments on days 1, 3 and 5 in 21-day cycles (Pietras and Hanahan, 2005; Bell-McGuinn et al., 2007). The total dosages for these optimized cyclophosphamide treatment schedules would be 315-420 mg/kg and 420 mg/kg for the LDM and MTD approaches, respectively. Therefore, cumulative doses received by LDM and MTD schedules in mice are comparable, as they are with humans, over a constant length of time.

In order to test whether or not the scheduling of cyclophosphamide administration can influence ovarian function, the total amount of drug received by our mice at the treatment
endpoint needed to be comparable as well. While conventional LDM and MTD treatments with cyclophosphamide use similar amounts of drug over a fixed period of time, the short estrous cycle of the mouse makes it difficult to compare the short-term scheduling effects on ovarian function while keeping treatment length constant. For example, in the time it would take for LDM treated mice to receive the same total dose used in a single MTD cycle, the ovaries of MTD treated mice could potentially have undergone multiple cycles of follicle development and be replenished by a completely new population of growing follicles (Pederson, 1970). Since MTD cyclophosphamide involves a 3 week period of rest, women of reproductive age will typically receive one dose of chemotherapy per menstrual cycle. Therefore, to model the effects of MTD cyclophosphamide administration on ovarian function in mice, which have 4 to 6-day estrus cycles, MTD doses were administered at 100 mg/kg every 6 days for a total of 2.5 chemotherapy cycles, while LDM treated mice received 20 mg/kg daily. This better modeled follicle exposure to chemotherapy while allowing total treatment dose and length to be held constant. As a result, the MTD dose was relatively low; however, single cyclophosphamide doses in the range of 75 to 150 mg/kg are most commonly used in studying the effects of MTD chemotherapy on mouse ovaries (Petrillo et al., 2011; Meirow et al., 2001; Kalich-Philosoph et al., 2013). It was also important that the treatment doses would not completely sterilize the mice, and a single 100 mg/kg dose has been shown to reduce primordial follicle numbers in C57Bl/6 mice by approximately 50% (Plowchalk and Mattison, 1992).

**Ovarian Damage Caused by Cyclophosphamide Exposure is Schedule-Dependent**

Mouse ovarian cycles were synchronized using a standard mouse superovulation protocol that involved hormonal treatments of PMSG and hCG (Luo et al., 2011). Pregnant mare serum gonadotropin is used to mimic the action of FSH in stimulating preovulatory follicular development, and is followed by hCG 48 hours later to induce ovulation. Ovaries were collected at time points throughout this cycle when stimulated follicles would presumably be in preantral, antral, and ovulatory, early luteal or luteal morphological stages. At these time points, ovarian weights were unchanged by cyclophosphamide treatment.

**Maximum-Tolerated Dose chemotherapy increased follicle recruitment and atresia**

Follicle counts revealed significant differences in preantral follicle numbers in mouse ovaries exposed to cyclophosphamide chemotherapy. The preantral follicle population was initially reduced shortly after MTD cyclophosphamide and then increased during the follicular phase, while at the same time, control and LDM preantral numbers decreased. These observations suggest that the MTD drug effects triggered an additional wave of development, of which the majority of follicles underwent atresia by the time ovulation was induced. In contrast, ovaries of LDM treated mice displayed similar follicle dynamics to control mice despite the
presence of fewer follicles at the initial stages. The LDM ovaries also appeared to have a smaller growing follicle population throughout the remaining stages, but the high variability of the control group did not allow this difference to be detected. Interestingly, the average preantral follicle count in the LDM treatment group was much less variable. This could be indicative of reduced competition during follicle selection as a result of decreased follicle numbers, since it was also found that both LDM and control ovaries had the same ratio of early growing to atretic follicles.

Conversely, the proportion of atretic follicles was significantly higher in ovaries of MTD treated mice during this ovarian cycle compared to the other treatment groups. Increased follicle atresia was measured immediately after MTD chemotherapy exposure and was consistent with levels reported in a recent dose-response trial of MTD cyclophosphamide exposure in mice (Ezoe et al., 2014).

An increase in growing follicle numbers in response to MTD cyclophosphamide has been previously documented in the literature. In fact, over 4 decades ago it was reported that a single 50 mg/kg dose of cyclophosphamide causes superovulation in mice 100 hours after administration (Russell et al., 1973). In another mouse study, following an initial decline in growing follicles triggered by a single 200 mg/kg dose of cyclophosphamide, there was a greater number of both preantral and antral follicles relative to controls 7 days after treatment (Plowchalk and Mattison). More recently, a high number of small growing follicles were detected in mouse ovaries 3 days after a single 150 mg/kg exposure to cyclophosphamide (Kalich-Philosoph et al. 2013). This wave of follicle recruitment was correlated with a decrease in the ovarian reserve that could not be attributed to direct primordial follicle damage based on TUNEL staining for apoptosis. Therefore, it was proposed that cyclophosphamide-induced ovarian damage was primarily a consequence of a primordial follicle burnout effect through increased follicle activation and loss (Kalich-Philosoph et al., 2013). Enhanced follicle activation can be explained by various potential mechanisms, since a delicate balance of inhibitory and stimulatory factors is responsible for maintaining primordial follicle dormancy and the controlled activation and recruitment to the growing follicle population. The rate of follicle activation can be influenced by the growing follicle population, which actively secrete suppressive factors such AMH. Anti-Müllerian hormone is produced by granulosa cells of primary to small antral follicles and has an inhibitory effect on follicle activation (Durlinger et al., 2002). Decreased levels of AMH due to an acute loss of small follicle numbers may, therefore, stimulate increased primordial follicle recruitment to the growing follicle pool. Interestingly, we did not see an increase in follicle recruitment during continuous LDM exposure of the drug, although there was a significant reduction in small follicles. It has also been proposed that cyclophosphamide could be capable of depleting the ovarian reserve by directly promoting the PI3K/PTEN/Akt pathway responsible for primordial follicle activation (Kalich-Philosoph et al., 2013). This hypothesis may help provide an explanation to the lack of follicle recruitment after LDM cyclophosphamide treatments, as low doses may not be sufficient
for direct follicle activation.

The follicle burnout effect could explain why MTD mice did not experience a reduction in successful ovulations based on luteal numbers, despite increased follicle atresia. In another study, corpora lutea numbers varied depending on the time point of tissue collection after MTD treatment. Following 75 mg/kg treatments of cyclophosphamide in mice, they actually discovered an increase in corpora lutea after 7 days (Plowchalk and Mattison, 1992). Variabilities in corpora lutea numbers are likely related to the fluctuations in follicle numbers in response to intermittent MTD drug delivery.

Taken together, our findings suggest that MTD chemotherapy is associated with enhanced follicle wasting through increased follicle recruitment and subsequent atresia. However, this does not appear to be a mechanism of LDM induced follicle loss.

Low-Dose Metronomic chemotherapy reduced growing follicle numbers and atresia

To date, very little is known about the long-term effects of LDM chemotherapy on fertility. Optimal anti-angiogenic doses still need to be determined for treatment in humans and it could be decades before the long-term effects of LDM chemotherapy are apparent. However, continuous low dose administration of cyclophosphamide is also used for maintenance immunosuppression in high-risk patients with system lupus erythematosus (SLE). The LDM treatment protocol for SLE involves daily oral cyclophosphamide in the range of 1-2 mg/kg (37-74 mg/m²) for a period of 6 to 9 months. In a clinical study that evaluated the long-term fertility outcomes of females treated with either schedule of cyclophosphamide for SLE, the LDM treatment regimen was not associated with a significant increase in the relative risk of permanent amenorrhea compared to IV pulse cyclophosphamide (1000 g/m², once a month for 6 months followed by 6 pulses every 3 months), despite the fact that greater cumulative doses had been received by patients that underwent the continuous protocol (LDM: 16.0±9.9 grams versus MTD: 9.07±4.3 grams) (Mok et al., 2006).

Following 2 weeks of LDM cyclophosphamide, our mice displayed a reduction in growing follicles, but the relative proportions of each growing follicle type was unchanged compared follicle populations sizes in untreated mice. Our findings are consistent with another recent report that mice exposed cyclophosphamide by a metronomic-like schedule (8 treatments of 50 mg/kg on alternate days) have fewer follicles at both 3 days, and 1 week post-treatment (Ezoe et al., 2014). We also found that follicle atresia was reduced, but still proportional, to the size of the growing follicle population ovaries LDM treated mice. Interestingly, luteal quantification did not indicate that the effects of LDM chemotherapy influence the total number of successful ovulatory events. Overall, it appears that LDM cyclophosphamide has the opposite short-term effect on ovarian follicle development compared to MTD cyclophosphamide based on our morphometric
analysis, since both follicle numbers and atresia were reduced by LDM chemotherapy, but increased by MTD chemotherapy. In contrast, ovaries of rats that had been given LDM cyclophosphamide (5 mg/kg/day) for a longer treatment length of 6 weeks were found to have a greater number of medium and large follicles relative to controls (Letterie, 2004). These observations are somewhat surprising considering LDM chemotherapy is anti-angiogenic and the fact that larger follicles are dependent on angiogenesis for survival, but could perhaps be an indication of slowed follicle growth during antral follicle development, which has previously been reported after administration of an anti-angiogenic drug in primates (Zimmerman et al., 2002). Existing studies, including our own, have only demonstrated short-term effects of LDM chemotherapy on the ovary and it remains unclear as to how these effects translate to long-term damage and fertility outcome compared to MTD scheduling.

**Mechanisms of Ovarian Damage**

Further analysis of both the murine and cell culture models were performed to provide insight into the underlying mechanisms of follicle loss due to LDM and MTD cyclophosphamide. The reduction in SIGC viability in vitro by 4HC exposure supports a granulosa cell damage-related mechanism of ovarian follicle loss. Reduced cell viability may be a consequence of a decelerated proliferation rates or increased apoptosis. In addition to a direct effect on follicular cells, it is possible that the decline in follicle numbers reflects an indirect consequence of damage to the follicle-supporting ovarian vasculature. Therefore, these potential mechanisms were explored using Western blot and immunofluorescence analyses of our isolated granulosa cell model, and by immunohistochemical analysis of ovarian tissue following our murine model of chemotherapy scheduling with cyclophosphamide.

* Rates of granulosa cell proliferation were altered by cyclophosphamide exposure

We performed immunostaining of Ki67 on mouse ovarian tissue at each stage of follicle development as a marker of proliferation after in vivo exposure to LDM and MTD chemotherapy. During the follicular phase, we did not detect a difference in the proportion of proliferating cells in either chemotherapy treatment group relative to the controls. However, Ki67-positive staining was significantly increased during the ovulatory and early luteal stages in ovarian sections of MTD and LDM treated mice, respectively. Increased cellular proliferation has been previously been reported in mouse ovaries 3 days after MTD chemotherapy exposure and was believed to be a reflective of the increase in the population of small growing follicles that also occurred at this time (Kalich-Philosoph et al., 2013). While this association can be made for our MTD treatment group, it is more difficult to explain the increased proliferation in the LDM treatment group during the luteal stages, especially since the mice received a metronomic dose of chemotherapy 24 hours prior to this time point.
When the effects of LDM and MTD were evaluated in vitro by PhosphoHistone-H3 immunofluorescence, SIGC proliferation was decreased by MTD treatment. Granulosa cell proliferation and differentiation is necessary for dominant follicles to survive in an environment that promotes follicular atresia of the remaining follicles during the process of follicle selection (Markström et al., 2002). Although we did not see a reduction in proliferation in our mouse model, it is possible that it contributed to the initial loss of follicles that was detected at our earliest time point after MTD cyclophosphamide. The newly recruited wave would not have been exposed to cyclophosphamide during its growth phase, which could be why the drug reduced cell proliferation in vitro, but not in vivo.

Maximum-Tolerated Dose chemotherapy caused high levels of granulosa cell DNA damage and apoptosis

Increased follicle atresia may have also been a response to direct drug-induced damage and subsequent apoptosis of follicular cells. Therefore, we evaluated levels of apoptosis following LDM and MTD treatments using Cleaved caspase-3 as a marker in vitro and in vivo. When measured in vivo by immunohistochemistry, expression of Cleaved caspase-3 was not significantly different in follicles of LDM mice relative to the controls. In contrast, Cleaved caspases-3 levels were elevated in MTD ovaries at the preantral and ovulatory stages of follicle development, which correlated to the time points when healthy follicle numbers declined. Expression of Cleaved caspase-3 was primarily localized to the granulosa cells of both preantral and antral follicles. Considering the high mitotic index of granulosa cells, which would make them particularly susceptible to the damage induced by cyclophosphamide (Plowchalk et al., 1992), this was not surprising.

The natural fate of 99% of follicles is regression by follicular atresia, which involves programmed granulosa cell death by apoptosis (Manabe et al., 2002). Therefore, increased Cleaved caspase-3 may precede and initiate follicle atresia or could be a sign of follicle atresia that had been triggered by some other mechanism. In order to determine whether the elevated Cleaved caspase-3 levels were potentially a direct result of cellular damage induced by cyclophosphamide, we looked for an association between DNA damage and Cleaved caspase-3 levels in isolated granulosa cells at various time points throughout LDM and MTD chemotherapy schedules in vitro.

Western blot and immunofluorescence analysis of granulosa cells treated with 4HC schedules revealed a greater increase in apoptotic cells after MTD chemotherapy compared to the equivalent dose administered by LDM scheduling by the end of the 6-day treatments. The MTD treated granulosa cell population also displayed a high proportion of cells with altered nuclear morphology, whereas only a few cells in the LDM treatment group displayed nuclear
irregularities. The abnormal nuclei were indicative of DNA damage or mitotic catastrophe (Filippi-Chiela et al., 2012), based on the presence of nuclei that were abnormal in size (large and small), irregular in shape and the presence of micronuclei that occasionally stained positive for Cleaved caspase-3. These nuclear alterations can be induced if a cell undergoes mitosis despite high amounts of DNA damage (Filippi-Chiela et al., 2012).

To detect cyclophosphamide-induced DNA damage, γ-H2AX immunofluorescence was measured at various time points throughout the in vitro chemotherapy schedules. Gamma-H2AX is the product of histone H2AX phosphorylation at Ser 139 due to the presence of DNA double strand breaks (DSB). Western blot analysis revealed that ubiquitinated γ-H2AX (ub-H2AX) is also recognized by the antibody we had visualized by immunofluorescence. Therefore, γ-H2AX levels determined by densitometric analysis were quantified as the sum of both ub-H2AX and γ-H2AX bands. Detection of ub-H2AX marks downstream signaling of γ-H2AX foci formation in the DNA damage repair pathway that leads to the accumulation of repair proteins at DSB sites (Bonner et al., 2008). Alternatively, γ-H2AX expression can lead to apoptosis if the damage is beyond repair. Differences in total γ-H2AX were consistent with levels of apoptosis in each treatment group. There were significantly higher levels of DNA damage at both 126 and 144 hours (54 and 72 hours after last 4HC exposure) of MTD treatment, and at the 144 hour time point (24 hours after last 4HC exposure) of LDM treatment. Gamma-H2AX expression peaks in oocytes around 12 to 24 hours after exposure to the cyclophosphamide metabolite, PM, and declines by 48 hours (Petrillo et al., 2011). Therefore, our treatment endpoint likely reflects peak γ-H2AX in LDM treated cells compared to persistent damage in MTD treated cells. Furthermore, dead cells were not included in either Western blot or immunofluorescence analyses, which were much more prevalent in MTD cultures. Even still, γ-H2AX levels were highest in MTD treated granulosa cells. These levels of γ-H2AX, along with the non-significant decrease in cell survival promoting factor Bcl-2 after MTD treatment, suggest that granulosa cell DNA damage could contribute to granulosa cell apoptosis and ultimately follicle atresia.

Maximum-Tolerated Dose chemotherapy interfered with normal regulation of ovarian angiogenesis

Follicles at every stage of development rely on the ovarian vasculature for survival and growth. Primordial and primary follicles are supported by nearby vessels within the ovarian stroma by passive diffusion. These vessels do not change during the reproductive cycle; however, the capillary network supporting the larger growing follicles increases in density within the theca layer as follicle development progresses, by a process known as ovarian angiogenesis (Suzuki et al., 1998). Hence, with each cycle, much the ovarian vasculature undergoes cyclic changes in blood vessel proliferation and regression. The dynamic nature of the ovarian
vasculature offers an additional opportunity for chemotherapy to target various stages of follicle and luteal development and consequently interfere with normal ovarian function. There is accumulating evidence in the literature of chemotherapy-induced damage to vessels that support primordial follicles (Meirow et al., 2007), growing follicles (Ezoe et al., 2014), as well as luteal structures (Ezoe et al., 2014). Data is currently limited, however, with regards to the effects of LDM chemotherapy on the ovarian vasculature. Being the “anti-angiogenic” chemotherapy strategy, we anticipated that LDM scheduling would interfere with normal ovarian angiogenesis to a greater extent than the MTD schedule, and could perhaps explain the LDM-induced reduction in growing follicle numbers. To compare the effects of these treatment schedules on ovarian function, we performed immunostaining for pro-angiogenic factor VEGF, anti-angiogenic factor TSP-1, and their respective receptors. Staining was quantified based on follicle type and status (healthy preantral, healthy antral and atretic) at each time point of tissue collection. Additionally, we measured blood vessel density using CD31 as an endothelial cell marker to visualize mature vessels.

Low-dose metronomic chemotherapy is known to exert anti-angiogenic effects by enhancing TSP-1 expression, which in turn suppresses levels of pro-angiogenic factor, VEGF, and its receptor (Gupta et al., 1999; Bocci et al., 2003; Zhang et al., 2009). Thrombospondin-1 was primarily expressed in granulosa of preantral, antral and atretic follicles. Follicles exposed to LDM and PBS treatments displayed a similar expression pattern to what has been previously reported for rats (Petrik et al., 2002), as TSP-1 peaked at preantral and early antral stages, and again during the luteal development phase. We observed the highest TSP-1 expression in atretic follicles, which positively reflects its role in promoting granulosa cell apoptosis and inhibiting angiogenesis (Garside et al., 2010). Follicles of MTD treated mice displayed a markedly different expression pattern than mice that received PBS or LDM cyclophosphamide. Interestingly, TSP-1 levels in all treatment groups displayed a strong inverse relationship with healthy preantral follicle numbers quantified at each stage of development, with the exception to the healthy antral follicles exposed to MTD chemotherapy (likely due to low statistical power; n<3).

The anti-angiogenic and apoptotic effects of TSP-1 in the ovary are mediated through binding membrane-associated CD36 receptors that are present on endothelial, granulosa and theca cells (Petrik et al., 2002). Temporal expression patterns of CD36 reflected that of TSP-1 in LDM and PBS treatment groups, with peak expression of both factors occurring at the antral stage of follicle development. In follicles from the MTD treatment group, however, CD36 expression was significantly elevated at the preantral and ovulatory stages of development. This may be related to its role in initiating apoptotic signaling upon TSP-1 binding, since these were the two stages that displayed major reductions follicle numbers, increased expression of Cleaved caspase-3 and high levels of follicle atresia. Activation of CD36 induces endothelial cell death and
inhibits neovascularization through the CD36-Fyn-caspase apoptotic cascade (Jiménez et al., 2000). While the inhibition of ovarian angiogenesis could indirectly lead to follicular regression, TSP-1 may also directly induce granulosa cell apoptosis through CD36 signaling that triggers Caspase-3 activation (Garside et al. 2010).

Our in vitro data also supported the possible activation of the TSP-1 mediated mechanism of cell death in granulosa cells when 4HC was delivered by MTD scheduling. There was an increase in TSP-1 from conditioned media of MTD treated cells that was considered non-significant, perhaps due to the daily media changes and the presence of these factors in the serum supplemented to the media of all groups. In addition, both CD36 and phosphorylated (activated) VEGFR-2 were down-regulated in MTD treated cells. The associated decline in activated VEGFR-2 can be explained by the ability of TSP-1 to directly bind and internalize VEGF (Greenaway et al., 2007), and antagonize VEGF activity at the receptor level (Zhang et al., 2009).

Theca and granulosa cells secrete increasing concentrations of VEGF (Barbaroni et al., 2000; Greenaway et al., 2004) during follicular development to promote follicular microvascularization. During our stimulated wave of follicle development, we observed higher levels of VEGF in healthy antral follicles compared to preantral follicles as expected. Furthermore, the temporal expression pattern of VEGF was inversely correlated to TSP-1 and positively reflected the pattern of fluctuation in follicle numbers during the follicular stages in each treatment group. Therefore, increased VEGF expression in preantral and antral follicles from the MTD treatment group at the antral stage was likely associated with the expanding population of small follicles in need of vascular support.

When preantral and atretic follicles both expressed increased VEGF levels at the antral stage of MTD treatment, they displayed differences in the expression of FLK-1 (VEGFR-2). This receptor was significantly upregulated in preantral follicles and downregulated in atretic follicles relative to controls. The difference between FLK-1 expression in preantral and atretic follicles could be related to follicle status. Thrombospondin-1, which can promote follicle atresia through follicular cell apoptosis and anti-angiogenesis, is known to downregulate FLK-1 as a result of co-clustering on the cell surface with the CD36 receptor (Zhang et al., 2009). Elevated TSP-1 in atretic follicles at the preceding time point (preantral stage) may therefore explain the reduction in FLK-1 at the antral time point in atretic follicles. Likewise, VEGF activity has been shown to reduce the expression of CD36 receptors (Thomas et al., 2008), and peak VEGF levels at the ovulatory stage preceded declined follicular CD36 expression at the luteal stages in all of our treatment groups.

The interactions among VEGF, TSP-1 and their receptors help to maintain their inverse relationship throughout the ovarian cycle. In our study, this relationship was evident in all follicle
types exposed to LDM and PBS treatments; however, there appeared to be a disruption in the balance in all follicle types exposed to MTD chemotherapy. For example, both TSP-1 and VEGF were elevated at the ovulatory stage in MTD ovaries relative to the other groups. Despite the apparent dysregulation of ovarian angiogenesis and increased follicle atresia in this group, corpora lutea numbers indicated that these effects did not interfere with the number of successful ovulatory events. It could be possible that the alterations in the regulation of ovarian angiogenesis following chemotherapy treatment help to uphold a successful ovarian cycle. For example, there were significantly higher levels VEGF in healthy antral follicles exposed to LDM and MTD chemotherapy relative to the controls, which could be an indication of poor follicle vascularization since hypoxia can induce VEGF expression. This may encourage enhanced antral follicle survival in the presence of chemotherapy-induced damage, as it was previously demonstrated in our lab that VEGF promotes granulosa cell survival in an apoptotic environment (Greenaway et al., 2004).

We measured ovarian blood vessel density 24 hours after exposure to LDM and MTD chemotherapy using CD31 as an indicator of mature blood vessels. There appeared to be a decrease in blood vessel density in ovarian sections exposed to MTD chemotherapy compared to PBS and LDM treated ovaries; however, this was not considered statistically significant. The use of CD31 staining was previously reported to underestimate vascular damage by chemotherapy, since it was demonstrated that the endothelial cell lining of large blood vessels can be preserved despite clear evidence of vascular damage in ovaries of women exposed to MTD chemotherapy (Meirow et al. 2007). Although vascular endothelial cells are considered the main targets for LDM chemotherapy, the mean ovarian vessel density of our LDM treated mice was similar to the controls.

We had expected the anti-angiogenic schedule to be more damaging to the ovarian vasculature, but our findings and existing evidence in the literature suggest that MTD chemotherapy has a greater negative effect on ovarian angiogenesis and the stromal vasculature than LDM chemotherapy. Visualization of newly formed blood vessels using CD34 staining allows detection of the microvasculature within the theca layer as early as the antral stage of follicle development as well as in the developing corpus luteum (Suzuki et al., 1998). Recently, Ezoe and associates evaluated chemotherapy-induced vascular damage based on CD34 positive staining in follicles and corpora lutea after exposure to increasing doses of cyclophosphamide in mice (Ezoe et al., 2014). They measured a dose-dependent decline in CD34 positive cells in developing follicles and luteal structures with MTD administration of the drug. Consistent with our CD31 data, the reduction in CD34 positive cells was greater after MTD scheduling than when the equivalent dose administered by LDM chemotherapy. Current evidence therefore suggests that
MTD chemotherapy schedules of cyclophosphamide are actually more detrimental to both mature and developing blood vessels of the ovary than LDM schedules.

In summary, our vascular data suggest that MTD cyclophosphamide scheduling interferes with normal ovarian angiogenesis to a greater extent than the equivalent LDM administration of the drug in vivo. The differences in expression of vascular factors following MTD chemotherapy were related to the number of growing follicles and the amount of follicular atresia, and varied depending on the time point, follicle type and status. To our surprise, neither our in vivo nor in vitro models demonstrated any changes in these factors in the ovarian cells following LDM chemotherapy scheduling of the drug.

Study Limitations

The major limitation of this study was the availability of tissue. Upon tissue collection, one ovary from each mouse was formalin fixed for immunohistochemical analysis and follicle counts while the other was flash frozen for protein collection. With only one ovary available for histological analysis, some ovarian sections needed to be shared for both immunohistochemical analysis and follicle quantification. Blood vessel staining by CD31 immunohistochemistry was not optimal on paraffin embedded ovarian sections due to high levels of background staining. Additionally, after finding that the expression of most of the vascular factors varied depending on the type of follicle being measured, it was presumed that it would be difficult to draw conclusions based on protein levels in whole ovary lysate. Analysis based on images of immunohistochemical stains at low magnification confirmed this. For these reasons, tissue that had been flash frozen were embedded in OCT and cryosectioned for blood vessel density quantification by immunofluorescence. This protocol allowed for more preserved tissue antigenicity and improved blood vessel staining with CD31; however, there were many artifacts likely due to the method of freezing and this tissue could not be used for additional follicle counts.

Our time points of tissue collection and limited tissue sections available did not allow us to assess both short and long-term effects of the drug on the ovary. Primordial follicle numbers were not included in the follicle counts due to their poor visibility in the H&E and immunohistochemical stains used to quantify the growing follicle population. We had originally planned to estimate primordial follicle availability using AMH levels in the serum, which is believed to be the most reliable marker of the ovarian reserve (La Marca et al., 2010). Granulosa cells of preantral to early antral follicles secrete AMH which acts to prevent new follicle recruitment. Therefore, reduced AMH levels are associated with diminishing ovarian reserve and the onset of menopause. In our model, we collected tissue immediately after chemotherapy exposure and measured fluctuation in preantral follicle numbers in the MTD treatment groups. We believed this would lead to abnormal AMH expression levels at the time points that serum was
collected and would inaccurately represent the ovarian reserve. For example, the initial decline in preantral follicles immediately following MTD cyclophosphamide would lead to a decline in AMH that underestimates ovarian reserve in comparison to the other groups. Furthermore, when this led to increased follicle recruitment and there were a large number of small growing follicles within this group, AMH levels would also increase and consequently overestimate the true primordial follicle population. Serum collection should therefore be taken at later time points (weeks or months after chemotherapy) so that follicle dynamics are stabilized and allow a better estimation of follicle reserve.

**Future Directions**

By evaluating the effects LDM and MTD administration of cyclophosphamide on ovarian function, we have demonstrated differences in short-term damage caused by chemotherapy scheduling on the ovary. Future directions should focus on how these effects contribute to long-term damage using methods to assess the ovarian reserve. This could be estimated based on primordial follicle counts of serial sectioned ovaries using a stain such as PCNA to enhance the visibility of primordial follicles (Mushelishvili et al., 2005; Picut et al., 2008). Tissue could be collected at later time points during the reproductive life span of the mouse and hormonal markers such as AMH could be also be used to estimate ovarian reserve. In addition, a copulatory breeding experiment should be performed in rodents in order to assess the reproductive outcome of the potential long-term ovarian damage from chemotherapy, based on the length of time or number of attempts before successful pregnancy, and litter size.

Measurement of CD34 or α-SMA in the developing follicle could provide a better indication of our treatments’ effects on ovarian angiogenesis, as they stain newly formed blood vessels in the ovary. Serum levels of FSH and LH as well as follicle-secreted factors such as E2 and inhibin B could also provide further insight to the dysregulation of ovarian function that was observed, especially following MTD schedules of cyclophosphamide.

Lastly, the results of this study demonstrate mechanisms of ovarian damage that may be specific to the drug cyclophosphamide. Future studies should compare the short- and long-term effects of LDM and MTD administration of various other chemotherapy drugs used to treat cancer to determine whether or not scheduling can influence their effects on ovarian function and fertility as well.
SUMMARY AND CONCLUSIONS

This study compared the ovarian effects of LDM and MTD chemotherapy scheduling in vitro and in vivo using 4HC and cyclophosphamide, respectively.

When subjected to 4HC scheduling in vitro, granulosa cell viability was unchanged by continuous exposure to anti-angiogenic levels of the drug. Concentrations in the range of achievable serum levels by MTD administration of cyclophosphamide in humans reduced granulosa cell viability. Furthermore, continuous low dose administration of the effective MTD dose of 4HC left cells significantly more viable than those receiving high intermittent doses. This demonstrated that the effects of 4HC are schedule-dependent in vitro and suggested that LDM scheduling would be less toxic to ovarian cells than MTD chemotherapy. In agreement with the measured viability levels, MTD scheduling of the drug was associated with higher levels of DNA damage, apoptosis and decreased proliferation in isolated granulosa cells.

The effects of cyclophosphamide on follicular granulosa cells could be responsible for the high levels of apoptosis and atresia in mouse ovaries exposed to MTD chemotherapy. Interestingly, the initial loss of follicles in the MTD group was followed by an increase in small follicles and a second wave of follicle atresia. Therefore, it appeared that MTD chemotherapy was associated with follicle wasting through increased recruitment. In contrast, mouse ovaries exposed to LDM chemotherapy had fewer growing follicles with proportional levels of atresia relative to controls. Unexpectedly, stromal blood vessel density and the expression of pro- and anti-angiogenic vascular factors were also similar to controls in LDM mouse ovaries. Conversely, expression patterns of these factors appeared suggestive of dysregulated angiogenesis in MTD mice.

In conclusion, we have shown that the ovarian effects of cyclophosphamide chemotherapy are schedule dependent, and overall, MTD chemotherapy causes a greater disruption of normal ovarian function than LDM scheduling. Long-term damage should be evaluated in order to determine how these short-term scheduling effects impact fertility.


Barisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R. Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during final growth of


Ellison LF, Wilkins K. Canadian trends in cancer prevalence. Health reports/Statistics Canada, Canadian Centre for Health Information. 2012;23:7-16


Fraser HM, Groome NP, McNeilly AS. Follicle-Stimulating hormone-inhibin B interactions during the follicular phase of the primate menstrual cycle revealed by gonadotropin-releasing hormone antagonist and antiestrogen treatment. J Endocrinol Metab. 1999 Apr;84(4):1365-9.


Holm K, Nysom K, Brocks V, Hertz H, Jacobsen N, Muller J. Ultrasound B-mode changes in the uterus and ovaries and Doppler changes in the uterus after total body irradiation and allogenic bone marrow transplantation in childhood. Bone Marrow Transplant. 1999 Feb;23(3):259-63.


Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL, Liosa V, Rueden C, Eliceiri KW, Carpenter AE. Improved structure, function and compatibility for CellProfiler:modular


Schipper I, Hop WC, Fauser BC. The follicle-stimulating hormone threshold/window concept examined by different interventions with exogenous FSH during the follicular phase of the normal menstrual cycle: duration, rather than magnitude of FSH increase affects follicle development. J Clin Endocrinol Metab. 1998 Apr;83(4):1292-8.


Thomas FH, Wilson H, Silvestri A, Fraser HM. Thrombospondin-1 expression is increased during


APPENDIX I – SOURCE OF PRODUCTS AND MATERIALS

0.1% Triton-X-100  
Sigma-Aldrich Canada Ltd., Oakville, ON

10% neutral buffered formalin  
Fisher Scientific, Whitby, ON

10x Phosphate buffered saline  
Life Technologies Inc., Burlington, ON
(PBS; cell culture)
3,3'-diaminobenzidine (DAB)  
Sigma-Aldrich Canada Ltd., Oakville, ON

30% acrylamide  
Bio-Rad, Mississauga, ON

30% Hydrogen peroxide solution  
Sigma-Aldrich Canada Ltd., Oakville, ON

4-15% polyacrylamide gradient gels  
Bio-Rad, Mississauga, ON

4-hydroperoxycyclophosphamide (4HC)  
Santa Cruz Biotechnology Inc., Santa Cruz, CA

4',6-diamidino-2-phenylindole (DAPI)  
Sigma-Aldrich Canada Ltd., Oakville, ON

AlexaFluor-488 conjugated anti-rabbit  
Life Technologies Inc., Burlington ON
IgG secondary antibody

AlexaFluor-594 conjugated anti-rabbit  
Life Technologies Inc., Burlington ON
IgG secondary antibody

Aluminum potassium sulfate  
Fisher Scientific, Whitby, ON

Amersham Hybond ECL nitrocellulose  
GE Healthcare Bio-Sciences Corp., Piscataway, NJ
blotting membrane

Ammonium persulfate (APS)  
Sigma-Aldrich Canada Ltd., Oakville, ON

Anti-γ-H2A.X antibody (ab11174)  
Abcam, Cambridge, MA

Anti-β-actin antibody (cs4967)  
Cell Signaling Technology, Danvers, MA

Anti-Bcl-2 (cs2876)  
Cell Signaling Technology, Danvers, MA

Anti-CD31 antibody (ab28364)  
Abcam, Cambridge, MA

Anti-CD36 antibody (NB400-144)  
Novus Biologicals, Oakville, ON

Anti-Cleaved caspase-3 antibody (cs9661)  
Cell Signaling Technology, Danvers, MA

Anti-Flk-1 antibody (sc6251)  
Santa Cruz Biotechnology Inc., Santa Cruz, CA

Anti-GAPDH antibody (cs2118)  
Cell Signaling Technology, Danvers, MA

Anti-Ki67 antibody (ab15580)  
Abcam, Cambridge, MA

Anti-PhosphoHistone-H3 (ab5176)  
Abcam, Cambridge, MA
Anti-TSP-1 antibody (sc81755)  
Santa Cruz Biotechnology Inc., Santa Cruz, CA

Anti-TSP-1 antibody (sc81755)  
Santa Cruz Biotechnology Inc., Santa Cruz, CA

Anti-VEGF antibody (ab46154)  
Abcam, Cambridge, MA

Anti-pVEGFR2 antibody (PA1-14441)  
Thermo Fisher Scientific, Nepean, ON

Antibiotic-antimycotic (ABAM)  
Life Technologies Inc., Burlington, ON

Aprotinin  
Sigma-Aldrich Canada Ltd., Oakville, ON

Biotinylated anti-mouse IgG secondary antibody  
Sigma-Aldrich Canada Ltd., Oakville, ON

Biotinylated anti-rabbit IgG secondary antibody  
Sigma-Aldrich Canada Ltd., Oakville, ON

Bovine serum albumin (BSA)  
Sigma-Aldrich Canada Ltd., Oakville, ON

Bromophenol Blue  
Sigma-Aldrich Canada Ltd., Oakville, ON

Cell culture flasks (T-75)  
Fisher Scientific, Whitby, ON

Citric acid  
Sigma-Aldrich Canada Ltd., Oakville, ON

Clarity Western ECL Substance  
Bio-Rad, Mississauga, ON

Cytoseal-XYL mounting media  
Thermo Fisher Scientific, Nepean, ON

Cyclophosphamide  
Sigma-Aldrich Canada Ltd., Oakville, ON

DC Protein Quantification Kit  
Bio-Rad, Mississauga, ON

Dithiothreitol (DTT; western blot)  
Life Technologies Inc., Burlington, ON

Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12)  
Life Technologies Inc., Burlington, ON

Ethanol (ETOH)  
Greenfield Ethanol Inc., Brampton, ON

Ethylenediaminetetraacetic acid (EDTA)  
Fisher Scientific, Whitby, ON

ExtrAvidin - Peroxidase  
Sigma-Aldrich Canada Ltd., Oakville, ON

Fetal bovine serum (FBS)  
Life Technologies Inc., Burlington, ON

Glycerol  
Fisher Scientific, Whitby, ON

Glycine  
Fisher Scientific, Whitby, ON

Hamilton syringe  
Fisher Scientific, Whitby, ON

Hematoxylin  
Fisher Scientific, Whitby, ON
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<td>Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody</td>
<td>Cell Signaling Technology, Danvers, MA</td>
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<td>Human chorionic gonadotropin (hCG)</td>
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<td>ImmEdge Hydrophobic Barrier Pen</td>
<td>Vector Laboratories, Burlington, ON</td>
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<td>Leupeptin</td>
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<td>Micro Cover Glasses, Round, 12mm</td>
<td>VWR International LLC, Mississauga, ON</td>
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<td>Nuclease-free microcentrifuge tubes (0.6mL, 1.5mL)</td>
<td>Fisher Scientific, Whitby, ON</td>
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<td>Optimal cutting temperature compound (OCT)</td>
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<td>Pepstatin A</td>
<td>Sigma-Aldrich Canada Ltd., Oakville, ON</td>
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<td>Phenylmethanesulfony fluoride (PMSF)</td>
<td>Roche Applied Science, Laval, QC</td>
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<td>Pregnant mare serum gonadotropin (PMSG)</td>
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<td>ProLong Gold Antifade Mountant</td>
<td>Life Technologies Inc., Burlington, ON</td>
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<td>Potassium iodate</td>
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<td>Potassium phosphate monobasic</td>
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<td>Precision Plus Protein Dual Color Standards</td>
<td>Bio-Rad, Mississauga, ON</td>
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<td>Serum Separator Tube (SST) BD Vacutainer plus blood collection tubes</td>
<td>Becton Dickinson and Company, Franklin, Lakes, NJ</td>
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<td>Sodium fluoride (NaF)</td>
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<td>Sodium potassium dibasic anhydrous</td>
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<td>Superfrost Plus glass slides</td>
<td>Fisher Scientific, Whitby, ON</td>
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<td>Tetramethylethylenediamine (TEMED)</td>
<td>Thermo Fisher Scientific, Nepean, ON</td>
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<td>Tetrasodium pyrophosphate</td>
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<td>Tris HCl</td>
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<td>Triton X-100</td>
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<td>Tween-20</td>
<td>Fisher Scientific, Whitby, ON</td>
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<tr>
<td>Water-soluble tetrazolium salt-1 (WST-1)</td>
<td>Roche Applied Science, Laval, QC</td>
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<tr>
<td>Xylene</td>
<td>Fisher Scientific, Whitby, ON</td>
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</tbody>
</table>
APPENDIX II – RECIPES FOR SOLUTIONS

20x Phosphate Buffered Saline (PBS)
Sodium chloride ................................................................. 60.0g
Potassium chloride ............................................................ 4.0g
Sodium potassium dibasic anhydrous .................................. 23.0g
Potassium phosphate monobasic ......................................... 4.0g
Reverse osmosis water (RO H₂O) ........................................ up to 1L

1x PBS
20x PBS (see recipe) ............................................................ 200mL
RO H₂O ........................................................................... 3.8L

1.0% Hydrogen Peroxide
30% Hydrogen peroxide solution ........................................... 6.6mL
1x PBS ............................................................................ up to 200mL

Citrate Buffer
Stock A – Citrate Buffer
Citric Acid ........................................................................... 2.1g
RO H₂O ............................................................... up to 100mL

Stock B – Citrate Buffer
Sodium citrate dihydrate ...................................................... 14.7g
RO H₂O ........................................................................... up to 500mL

Working Citrate Buffer
Stock A ............................................................................ 18mL
Stock B ............................................................................ 82mL
RO H₂O ........................................................................... up to 1L
pH to 6.0

Working Citrate with Tween-20 Buffer
Stock A ............................................................................ 18mL
Stock B ............................................................................ 82mL
RO H₂O ........................................................................... up to 1L
pH to 6.0 then continue
Tween-20 ........................................................................... 0.5mL

Blocking Solution (5% BSA/0.1% sodium azide)
BSA powder ......................................................................... 10g
Sodium azide ........................................................................ 0.2g
1x PBS .............................................................................. up to 200mL

Antibody Diluting Fluid (1% BSA/0.2% sodium azide)
BSA powder ......................................................................... 1.0g
Sodium azide ........................................................................ 0.02g
1x PBS .............................................................................. up to 100mL
Carazzi’s Hematoxylin
- Hematoxylin: 0.25g
- Glycerol: 50mL
- Aluminum potassium sulfate: 12.5g
- Potassium iodate: 0.05g
- RO H2O: 200mL

RIPA Lysis Buffer
- 10mM Tris HCl: 0.788g
- RO H2O: up to 495mL
- pH to 7.6 then continue
- 5mM EDTA: 0.7306g
- 50mM Sodium chloride: 1.461g
- 30mM Tetrasodium pyrophosphate: 3.988g
- 1% Triton X-100: 5mL

Protease Inhibitors (add fresh before each use)
- Per mL RIPA
  - Aprotinin: 2μL
  - Phenylmethanesulfony fluoride (PMSF; 0.871 g/mL): 20μL
  - Sodium Orthovanadate (NaV; 0.1 mM): 2μL
  - Sodium fluoride (NaF; 50.0 mM): 50μL
  - Pepstatin A (1 mg/mL): 1μL
  - Leupeptin (2 mg/mL): 1μL

3x Reducing Buffer
- 10% SDS: 2mL
- Glycerol: 1mL
- 1M Tris HCl: 0.5mL
- Tris HCl: 1.576g
- RO H2O: up to 10mL

Bromophenol Blue: 10mg
- RO H2O: up to 9mL
- Dithiothreitol (DTT): 1:7 ratio

12% Separating PAGE Gel
- RO H2O: 3.3mL
- 30% Acrylamide: 4mL
- 1.5M Tris buffer: 2.5mL
- Tris base: 18.16g
- RO H2O: up to 100mL

pH to 8.8
- 10% SDS: 0.1mL
- 10% Ammonium persulfate (APS): 0.1mL
- APS: 0.1g
- RO H2O: 1mL
- Tetramethylethylenediamine (TEMED): 8μL

Pour into gel mold, leaving space for stacking gel. Fill mold to top with RO H2O until gel resolved (See recipe for 5% Stacking PAGE Gel)

6% Separating PAGE Gel
- RO H2O: 5.3mL
- 30% Acrylamide: 2mL
1.5M Tris buffer ................................................................. 2.5mL
  Tris base ................................................................. 18.16g
  RO H₂O ................................................................. up to 100mL
  pH to 8.8
10% SDS ................................................................. 0.1mL
10% Ammonium persulfate (APS) .................................... 0.1mL
  APS ................................................................. 0.1g
  RO H₂O ................................................................. 1mL
Tetramethylethylenediamine (TEMED) ................................ 16μL
Pour into gel mold, leaving space for stacking gel. Fill mold to top with RO H₂O until gel resolved (See recipe for 5% Stacking PAGE Gel)

5% Stacking PAGE Gel
  RO H₂O ................................................................. 2.1mL
  30% Acrylamide ....................................................... 0.5mL
  1.0M Tris buffer ....................................................... 0.38mL
    Tris base .......................................................... 12.12g
    RO H₂O ................................................................. up to 100mL
  pH to 6.8
10% SDS ................................................................. 30μL
10% Ammonium persulfate (APS) .................................... 30μL
  APS ................................................................. 0.1g
  RO H₂O ................................................................. 1mL
Tetramethylethylenediamine (TEMED) ................................ 6μL
Pour into mold on top of separating gel. Place comb into mold and allow to resolve.

5x Running Buffer (Tris-Glycine Buffer)
  Tris base ........................................................... 15.1g
  Glycine ............................................................... 72.1g
  10% SDS .............................................................. 10mL
  RO H₂O ................................................................. up to 1L

1x Running Buffer (Tris-Glycine Buffer)
  5x Running buffer ................................................... 200mL
  RO H₂O ................................................................. 800mL

10x Transfer Buffer
  Tris base .......................................................... 30.2g
  Glycine ............................................................... 144g
  RO H₂O ................................................................. up to 800mL

1x Transfer Buffer
  Methanol ............................................................. 200mL
  RO H₂O ................................................................. up to 900mL
  10x Transfer buffer .................................................. 100mL

10x Tris buffered saline (TBS)
  Tris base ........................................................... 24.2g
  Sodium chloride ...................................................... 80.0g
  RO H₂O ................................................................. up to 1L
  pH to 7.6
1x Tris buffered saline with Tween-20 (TBST)
- 10x TBS (see recipe) ................................................................. 100mL
- RO H₂O ........................................................................ up to 1L
- Tween-20 ............................................................................ 1.0mL

5% BSA in TBST
- BSA 2.5g
- TBST .............................................................................. up to

5% Skim milk in TBST
- Skim milk powder .............................................................. 5.0g
- TBST ................................................................................ up to 100 mL
APPENDIX III – PRIMARY AND SECONDARY ANTIBODY OMISSION CONTROLS

Primary Omission Control (1'O): Staining without primary antibody

Secondary Omission Control (2'O): Staining without secondary antibody

Magnification used for quantification shown.

Immunofluorescence - Fixed cells:

<table>
<thead>
<tr>
<th>Antibody Solutions</th>
<th>1'O</th>
<th>2'O</th>
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</thead>
<tbody>
<tr>
<td>PhosphoHistone-H3</td>
<td>1': 1:2000</td>
<td>2': 1:100-R (594)</td>
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<tr>
<td></td>
<td>Blue: DAPI, Green: HH3</td>
<td></td>
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<tr>
<td>Cleaved Caspase-3</td>
<td>1': 1:400</td>
<td>2': 1:100-R (488)</td>
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<td>Blue: DAPI, Red CC3</td>
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<tr>
<td>Gamma-H2AX</td>
<td>1': 1:1000</td>
<td>2': 1:200-R (594)</td>
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<tr>
<td></td>
<td>Red: DAPI, Green: H2AX</td>
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**Immunofluorescence - Cryosections**

Antibody Solutions

<table>
<thead>
<tr>
<th>CD31 (200x)</th>
<th>1' 1:25</th>
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<td>2' 1:100-R (594)</td>
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<tr>
<td>Blue: DAPI, Red: CD31</td>
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**Immunohistochemistry - Citrate antigen retrieval on paraffin embedded sections:**

Antibody Solutions

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<th>Cleaved Caspase-3 (600x)</th>
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<td>2' 1:100-R</td>
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<table>
<thead>
<tr>
<th>Ki67* (600x)</th>
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<td>2' 1:100-R</td>
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**Immunohistochemistry - Citrate antigen retrieval (cont’d):**

<table>
<thead>
<tr>
<th>Antibody Solutions</th>
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<th>2˚O</th>
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<tbody>
<tr>
<td>CD36 (600x)</td>
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<td>1˚ 1:1000</td>
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<td>2˚ 1:100-R</td>
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<tr>
<td>VEGF (600x)</td>
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<td>1˚ 1:400</td>
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<td>2˚ 1:100-M</td>
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</table>

Antibody sources are detailed in full in Appendix I. *Antigen retrieval performed using Citrate Buffer supplemented with 0.05% Tween 20. R-rabbit secondary, M-mouse secondary. 594/488-secondary Alexa Fluor colour wavelength. Additional details in methods.
Input: Brown- DAB stain (protein of interest). Blue-haematoxylin counterstain

Output: Pseudo-coloured image showing analysis markup

Magnification used for quantification shown.

ImageScope

<table>
<thead>
<tr>
<th>Antibody Solutions</th>
<th>Input</th>
<th>Output</th>
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<td>VEGF (600x)</td>
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<td>Antibody Solutions</td>
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