Prevention of chemotherapy-induced ovarian damage: possible roles for hormonal and non-hormonal attenuating agents

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BACKGROUND: Current options for female fertility preservation in the face of cytotoxic treatments include embryo, oocyte and ovarian tissue cryopreservation. However these methods are limited by the patient age, status or available timeframe before treatment and they necessitate invasive procedures. Agents which can prevent or attenuate the ovotoxic effects of treatment would provide significant advantages over the existing fertility preservation techniques, and would allow patients to retain their natural fertility without the necessity for costly, invasive and risky
Introduction

One of the most significant long-term sequelae of exposure to cytotoxic drug treatments is infertility, secondary to premature ovarian failure (POF) or insufficiency. As a result, protection of the ovarian reserve and prevention of infertility has risen to the fore as the primary quality of life issue facing patients and their physicians. Current options for fertility preservation in female cancer patients include oocyte/embryo cryopreservation, and ovarian tissue cryopreservation. These methods are limited both in their scope (because they are only available to certain populations of patients) as well as by factors such as time, cost, and the invasive nature of the procedures. Agents that can prevent the loss of follicles at the time of treatment would provide significant advantages over existing fertility preservation techniques in that they would be suitable for patients of all ages and life stages, would not require invasive surgical procedures or subsequent use of assisted reproductive technologies, and would prevent the myriad endocrine related side effects of POF other than infertility.

Until recently, there had been limited progress in the field of preventative fertility preservation, largely due to our limited understanding of the mechanisms underlying the impact of cytotoxic drugs on the ovary. However, there have been a number of recent advances in this area and, with a clearer picture of how chemotherapy destroys the ovarian follicle reserve, there has been parallel progress in the development of preventative fertility preservation agents. This paper will outline the impact and mechanisms of cytotoxic drugs on the various cell-types of the ovary, and review the recent developments in the field of fertility preservation for female cancer patients.

Methods

This paper provides a review of the literature on the mechanisms of cytotoxic-induced ovarian damage and the implications for fertility preservation. PubMed was used to identify relevant articles published in English up to December 2013, utilizing the following terms: ‘fertility chemotherapy’, ‘chemotherapy ovarian failure’, ‘cancer infertility’, ‘prevention chemotherapy infertility’ and ‘mechanisms chemotherapy ovarian damage’. Relevant articles referenced within articles found in Pubmed were also reviewed based upon their relevance as determined by the authors.

Impact of cytotoxic drugs on ovarian function

Clinically, the impact of cytotoxic treatments on the ovary ranges from none, through varying levels of partial damage resulting in reduced fertility (Bath et al., 2003; Anderson et al., 2006), all the way to complete ovarian atrophy, total loss of follicle reserve and infertility (Himmelstein-Braw et al., 1978; Waxman, 1983; Meirow and Nugent, 2001). The degree of ovarian damage and resulting infertility depend on the type of drug and dose received, and patient age at treatment (Sklar et al., 2006; Meirow and Wallace, 2009), with older patients more likely to develop premature ovarian failure (POF) post-treatment. This is largely due to the reduction in ovarian reserve that naturally occurs with age; since older women have diminished primordial follicle stockpiles, the effects of chemotherapy-induced follicle destruction are more significant (Meirow et al., 2010; Anderson and Wallace, 2013) (Fig. 1).

Classes of cytotoxic drugs

There are seven main families of chemotherapeutic drug groups grouped by mode of action: alkylating agents, platinum derivatives, anthracycline antibiotics, plant alkaloids, antimetabolites, taxanes and biologic agents.

(1) Alkylating agents have extremely damaging effects on the ovary (Familiari et al., 1993; Ataya et al., 1995; Meirow et al., 1999); and are responsible for the highest age-adjusted odds ratio of ovarian failure rates, followed by other drug families (Meirow et al., 2010).

(2) Platinum-based compounds, such as cisplatin, carry a medium risk of amenorrhea (Maneschi et al., 1994; Meirow, 2000; Nozaki et al., 2009). Cisplatin has been shown to induce DNA damage in the ovary (Blommaert et al., 1996), possibly via c-abl tyrosine kinase and p63 activation (Gonfloni et al., 2009). Genetic effects on the oocyte result in early embryonic mortality and marked aneuploidy (Higdon et al., 1992).

(3) Anthracyclines such as doxorubicin (DXR) have been shown to induce oxidative stress, dominant lethal mutations and aneuploidy in maturing pre-ovulatory oocytes in female mice (Malhes, 1995). Clinically, their impact on fertility has variously been categorized as medium (Blumenfeld, 2012) and low risk (Meirow et al.,...
An early study reported the incidence of amenorrhea in women younger than 30 years old treated with doxorubicin was 0%, compared with 33% in women aged 30–39, and 96% for women aged 40–49 (Hortobagyi et al., 1986).

(4) Vinca alkaloids are aneuploidy inducing, and animal studies show high levels of aneuploidy in oocytes exposed to vinblastine (Mailhes, 1995). Clinical studies however document no increased risk of ovarian failure (Meirow, 2000; Lee et al., 2006).

(5) While there are limited data available on the effects of antimetabolites on the ovary, there are some indications that they do not impact on fertility. The addition of methotrexate and 5FU to alkylating agent regimens was not associated with an increase in amenorrhea post-treatment (Bines et al., 1996). Methotrexate is commonly used to treat ectopic pregnancy without any effect on subsequent fertility (Mol et al., 2008; Oriol et al., 2008).

(6) There are limited and conflicting data regarding the effects of the taxane drug family on ovarian failure rates. While many studies have reported low or no increased risk of amenorrhea (Davis et al., 2005; Reh et al., 2008; Han et al., 2009; Abusief et al., 2010; Ganz et al., 2011), other studies have documented gonadal toxicity with high FSH levels (Anderson et al., 2006), and an increase in the incidence of amenorrhea (Petrek et al., 2006; Han et al., 2009).

(7) Biological targeted therapies are a relatively new and growing category of anti-cancer treatments that derive from living organisms. These agents are designed to target very specific cells, toxic effects are unlikely. An additional indirect impact of these drugs on patient fertility relates to the nature of treatment. These drugs are typically given as adjuvant therapy for many years after the initial cancer treatments, and new recommendations advocate extending adjuvant treatment beyond the current 5 years (Higgins et al., 2013). Since they cannot be taken while pregnant, adjuvant therapies by necessity enforce a delay on any future attempts to conceive, resulting in an age-related decline in fertility.

Most commonly, patients are subjected to combinations of these drug families, and this, combined with individual patient variation, makes it difficult to predict patient risk of ovarian damage in advance. Certain combinations of chemotherapy have become standard treatment protocol for specific cancers, and there are data on ovarian failure rates induced by the more common regimes (Table I, Meirow et al., 2010). Among the less ovotoxic are combinations such as ABVD (adriamycin, bleomycin, vincreistine and dacarbazine) for lymphoma, which rarely results in premature ovarian insufficiency (POI) (Bonadonna et al., 2004; Decantar et al., 2010; Behringer et al., 2013). In contrast, the majority of lymphoma patients treated with COPP (cyclophosphamide, vincristine, procarbazine, prednisone) report ovarian failure (Kreuser et al., 1992; Behringer et al., 2005), as do 72–100% of patients who undergo high-dose multiple-agent chemotherapy in preparation for bone marrow transplantation (Chung et al., 2013). And while only 15% of patients receiving ACT (doxorubicin, cyclophosphamide and taxol) for breast cancer were amenorrheic >12 months after treatment (Formier et al., 2005; Sukumvanich et al., 2010), this can be a misleading end-point since those who demonstrate menstruation can show a significant
cause a reduction in ovarian weight in rats (Sato et al., 2007). These observations are supported by animal studies on the specific and are cytotoxic even when cells are at rest although proliferation (Mitchison, 2012), and as such the dormant oocyte in the primordial follicle is not a natural target. Alkylating agents are not cell-cycle specific and are cytotoxic even when cells are at rest although proliferating cells are known to be more sensitive to their effects (Dann et al., 2005).

Histological studies on human tissue show that chemotherapy causes a drastic loss of primordial follicle stocks (Nicosia et al., 1985; Marcello et al., 1990; Meirow et al., 1999; Oktem and Oktay, 2007). These observations are supported by animal studies on the major cytotoxic agents. The alkylating agent, cyclophosphamide, causes a reduction in ovarian weight in rats (Sato et al., 2009), diminished primordial follicle stockpiles in mice in a dose-dependent manner (Meirow et al., 1999) and has been associated with a reduction in primordial follicles in the rhesus macaque (Ataya et al., 1995). Paclitaxel and cisplatin have also been observed to decrease the number of primordial follicles in mice and rats (Yucebilgin et al., 2004; Gonfloni et al., 2009). This loss of follicles could be due to a direct effect of the treatment on follicles or an indirect effect via another cell type such as stroma.

**Mechanisms of chemotherapy-induced ovarian damage**

In general, cytotoxic treatments work by causing DNA damage, then triggering a complex cascade response in which the cell initially attempts DNA damage repair and failing that undergoes apoptosis. Persistent unrepaired DNA double-strand breaks (DSB) are thought to be among the types of DNA damage caused by chemotherapies that activate apoptotic death in oocytes (Di Giacomo et al., 2005; Jurisicova et al., 2006; Perez et al., 2007). Signaling pathways involved in both DNA repair and induction of apoptosis are finely regulated by post-translational modifications. Both the cell type and, in particular for germ cells, the cell cycle stage at the time of exposure will define the molecular response to cytotoxicity. Between the primordial and antral follicle stages, the oocyte is in the dictyate (prolonged diplotene) stage of prophase I arrest. Following the LH surge, the oocyte is triggered to enter into the first meiotic M-phase, M1 (Carroll and Marangos, 2013).

**Impact of cytotoxic treatments on the oocyte during growth**

Exposure of the oocyte during antral stages of growth to cytotoxic agents raises significant concerns if fertilization occurs shortly after exposure since female germ cell damage can impact fertilization and spontaneous abortion rates, as well as possibly lead to congenital abnormalities in offspring (Aronn et al., 2001; Meirow and Schiff, 2005). Animal studies have shown definitively that most cytotoxic drug classes are mutagenic and teratogenic when oocytes are exposed during maturation. In mice, alkylating agents are mutagenic to oocytes (Meirow et al., 2001) and cause increased rates of spontaneous abortions and fetal malformations (Becker and Schoneich, 1982; Meirow et al., 2001). Cisplatin and its

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<td>Diagnosis</td>
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AC, doxorubicin cyclophosphamide; CAF, cyclophosphamide doxorubicin and fluorouracil; CMF, cyclophosphamide, methotrexate and fluorouracil; ABVD, adriamycin, bleomycin, vincristine and dacarbazine; BEACOPP, bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisone; POF, premature ovarian failure; AMH, anti-Mullerian hormone.

The clinical picture, however, does little to explain the larger and more fundamental question of the mechanisms underlying the ovotoxicity of these drugs.

**Destruction of ovarian follicle reserve**

The impact of chemotherapy on fertility is directly dependent on the survival or loss of the dormant oocytes in the primordial follicles that comprise the ovarian follicle reserve. It is important to distinguish between the short and long-term effects of chemotherapy on the ovary. The immediate effect, occurring during treatment, is temporary amenorrhea as a result of the destruction of the growing follicles. Of greater long-term import is the damage caused to the primordial follicle pool. While total loss of the primordial follicle population can occur, and will result in immediate and permanent sterilization, more common is partial loss of the primordial follicle reserve. If enough primordial follicles remain, the amenorrhea induced by the loss of the growing follicle population will be short lived. Petrek et al. (2006) found that while 84% of women treated with doxorubicin and cyclophosphamide became amenorrheic during treatment, almost half recovered ovarian function within 9 months of cessation of treatment. However, the reduction of the primordial follicle pool decreases the remaining window of fertility available to the patient, resulting in permanent amenorrhea and premature menopause years or even decades after treatment (Sklar et al., 2006; Partridge et al., 2010; Gracia et al., 2012).

Most classes of cytotoxic drugs preferentially target rapidly dividing cells, interrupting essential cell processes and arresting cellular proliferation (Mitchison, 2012), and as such the dormant oocyte in the primordial follicle is not a natural target. Alkylating agents are not cell-cycle specific and are cytotoxic even when cells are at rest although proliferating cells are known to be more sensitive to their effects (Dann et al., 2005). Histological studies on human tissue show that chemotherapy causes a drastic loss of primordial follicle stocks (Nicosia et al., 1985; Marcello et al., 1990; Meirow et al., 1999; Oktem and Oktay, 2007). These observations are supported by animal studies on the major cytotoxic agents. The alkylating agent, cyclophosphamide, causes a reduction in ovarian weight in rats (Sato et al., 2009), diminished primordial follicle stockpiles in mice in a dose-dependent manner (Meirow et al., 1999) and has been associated with a reduction in primordial follicles in the rhesus macaque (Ataya et al., 1995). Paclitaxel and cisplatin have also been observed to decrease the number of primordial follicles in mice and rats (Yucebilgin et al., 2004; Gonfloni et al., 2009). This loss of follicles could be due to a direct effect of the treatment on follicles or an indirect effect via another cell type such as stroma.
analogs have been shown to induce dominant lethal mutations in female mice (Blommaert et al., 1995) and have been linked to an increase in embryonic mortality rates (Katoh et al., 1990; Higdon et al., 1992). Anthracycline antibiotics, which inhibit topoisomerase II activity, have been shown to induce lethal dominant mutations in maturing and pre-ovulatory oocytes in mice (Katoh et al., 1990). DXR exposure in vitro causes chromosomal disintegration in GV oocytes (Bar-Joseph et al., 2010) and, as one study suggests, multigenerational effects with increased rates of neonatal death, physical malformations and chromosomal abnormalities more than four generations after a single DXR exposure (Kujjo et al., 2011). Vinca alkaloids have been shown to cause aneuploidy in mouse and hamster oocytes (Russo and Pacchierotti, 1988; Tateno et al., 1995) and MI meiotic arrest in oocytes that have been observed to remain capable of fertilization, resulting in fetal abnormalities (Albanese, 1987). Concern has also been raised over the impact antimitabolites have on female germ cells; however, a retrospective study of women who conceived <6 months after MTX treatment for ectopic pregnancy found no increase in fetal malformations or adverse outcomes (Svirsky et al., 2009). Paclitaxel inhibits mitosis by stabilizing microtubules and has been shown to result in aneuploidy in mouse oocytes (Mailhes et al., 1999).

Despite the teratogenic effects chemotherapy has been shown to have in animal models, long-term follow-up studies on cancer survivors show no significant increases in fetal malformation or miscarriage after cytotoxic drug treatment (Green et al., 2002, 2009; Dalberg et al., 2006; Mueller et al., 2009), and no higher risk of genetic or chromosomal abnormalities in children born to cancer survivors compared with controls (Winther et al., 2004; Green et al., 2009). While this is reassuring, it is important to realize that these data are almost entirely acquired from women who became pregnant years after completing chemotherapy and thus refers to pregnancies derived from oocytes exposed as primordial follicles. The timing of oocyte exposure to cytotoxic treatment is decisive in terms of the risk of mutagenesis (Meirow and Schiff, 2005). In humans it takes ~6 months for an oocyte to fully mature (Gougeon, 1986; McGee and Hsueh, 2000) and as a result, pregnancies achieved >6 months post-treatment (such as those reported on in these studies) were generated from oocytes exposed in a dormant state, which therefore remained genetically undamaged. Current recommendations are to not allow patients to undertake embryo or oocyte cryopreservation between chemotherapy treatments and to wait at least 6 months post-chemotherapy before conception (Chung et al., 2013).

**Apoptosis and DNA repair pathways in growing follicles**

Cytotoxic drug-induced apoptotic pathways have been well documented in growing follicles (Perez et al., 1997; Utsunomiya et al., 2008). Apoptosis of growing follicles originates in the granulosa cells, and there is some evidence that it is mediated by the FasL transmembrane protein, a Fas antigen-specific death inducer (Utsunomiya et al., 2008). Within the oocyte, anthracycline agents such as DXR have been shown to induce chromosomal fragmentation as well as fragmentation of DNA.
of the cytoplasm into apoptotic bodies (Perez et al., 1997, 1999; Bar-Joseph et al., 2010). Mature oocyte apoptosis is mediated by several molecules including ceramide, Bax and the caspases (Fig. 2) (Perez et al., 1997; Morita et al., 2000; Depalo et al., 2003). Ceramide functions as a probable pro-apoptotic signal in mature oocytes (Morita et al., 1999) and has been identified as an initiator of DXR-induced apoptosis in mature oocytes (Morita et al., 2000). Bax, a protein produced by the Bcl2-gene family, plays an essential role in DXR-initiated apoptosis in mature mouse oocytes (Perez et al., 1997; Morita et al., 1999). Caspases, members of the CASP protease family, have been shown universally to play a pivotal role as cell death effector molecules, and are central in the apoptotic pathway of mature oocytes (Bergeron et al., 1998; Tilly, 1998; Morita et al., 1999). Caspases-2, -12 and -3 specifically have been shown to play a role in mature oocyte apoptosis (Takai et al., 2007; Bar-Joseph et al., 2010). Mature mouse oocytes pretreated with a caspase inhibitor as well as mature oocytes derived from mice that lack expression of caspase-2 show a marked resistance to DXR-induced apoptosis (Perez et al., 1997).

Rad51, a highly conserved protein necessary for homologous recombination-dependent DSB repair (Wu et al., 2008), is thought to play a critical role in oocyte resilience to DNA damage (Kuju et al., 2010; Kuijo et al., 2012). Chemosensitivity in mature oocytes has been highly correlated with the presence of Rad51 (Kuijo et al., 2010), and microinjection of recombinant Rad51 into mouse and bovine oocytes can decrease the extent of chemotherapy and radiation-induced DNA double-strand breaks and suppress apoptosis (Perez et al., 2007; Kuijo et al., 2012). Bax interferes with the binding of Rad51 to DNA and the repair of damaged DNA (Fig. 2), and inactivation of the Bax gene significantly reduces the number of oocytes which undergo apoptosis in response to chemotherapy (Perez et al., 1999, 2007).

There is also evidence that exposure to toxicants can induce follicle loss via non-apoptotic mechanisms, such as autophagy. A study which examined the ovaries of mice exposed to cigarette smoke showed no change in apoptotic indicators, but found an increased number of autophagosomes in granulosa cells of ovarian follicles together with increased expression of Beclin-1 and microtubule-associated protein light chain 3, key regulatory proteins in the autophagy pathway (Gannon et al., 2012). Whether autophagy plays a role in chemotherapy-induced follicle loss has not been examined.

Effects of cytotoxic treatments on dormant primordial follicles

The predominant effect of cytotoxic treatments on primordial follicles in vivo is either total or partial loss of these follicles. While apoptosis in mature follicles in response to chemotoxic exposure has been clearly demonstrated and the specific pathways have been investigated in depth, studies which have attempted to examine the effects of chemotherapy on primordial follicles have relied heavily on the xenograft model, which has inherent flaws. Human ovarian grafts transplanted into SCID mice then exposed to cyclophosphamide do demonstrate increased apoptotic cell death, followed by a rapid decline in primordial follicle number (Oktem and Oktay, 2007). However, the xenograft is not in all ways a true in vivo system since the transplanted ovarian pieces have been removed from paracrine growth factors responsible for suppression of follicle growth (Baird et al., 2004). And since there is some evidence that the process of transplantation itself induces follicle recruitment and growth (Gavish et al., 2012), it is possible that the apoptotic cell death seen in xenografts is actually occurring in non-dormant, early growing follicles. In a similar fashion, in vitro culture has been shown to trigger spontaneous primordial follicle activation in a number of animal species (Picton et al., 2008) and in human ovarian tissue (Telfer et al., 2008), thus any apoptosis seen in this model may be occurring in follicles which have already begun the maturation process; however this remains to be proven.

DNA damage and apoptotic pathways in primordial follicles

A number of in vivo irradiation studies have helped elucidate key factors in the apoptotic pathway in primordial follicles. Anti-oncogene p53 is a key mediator of DNA damage-induced apoptosis in somatic cells (Harris and Levine, 2005), and has been shown to be involved in apoptosis induction in rat granulosa cell lines (Zwain and Amato, 2001). However, studies have shown that human ovarian follicles do not stain for p53 (Depalo et al., 2003; Livera et al., 2008). Additionally, DXR treatment induced the same level of apoptosis in mature oocytes obtained from mice with a disruption of the p53 gene as in controls (Perez et al., 1997). It is therefore likely that DNA injury induces apoptosis independently of p53, and in fact, several studies have identified p63, a homolog of p53 found in the nucleus of oocytes, and specifically the TAp63-α isoform, as a key mediator of the DNA damage/repair pathways in the response of primordial follicle oocytes to DNA injury (Kurita et al., 2005; Suh et al., 2006; Livera et al., 2008). The p63 pathway is up-regulated when oocytes are exposed to external triggers of DNA damage such as radiation (Livera et al., 2008), and loss of p63 in mouse oocytes results in resistance to the apoptotic effects of radiation (Suh et al., 2006).

Pro-apoptotic proteins PUMA and NOXA (see Fig. 2) have been shown to play a key role in TAp63 mediated apoptosis of DNA damaged oocytes (Kerr et al., 2012). Oocytes without PUMA and/or both PUMA and NOXA are protected from γ-irradiation-induced apoptosis and can produce healthy offspring, indicating that the protected oocytes are capable of DNA repair and subsequent normal function. C-Abl tyrosine kinase has been shown to act as a ‘switch’ for TAp63 transcriptional activity and the apoptotic pathway following exposure to chemotherapy agents, cisplatin (Goni-Fonli et al., 2009) and DXR (Yoshida and Miki, 2005). Other studies have further demonstrated that C-Abl plays a role in the maintenance of genomic integrity by dealing with DNA breaks in both meiotic and mitotic cells (Kharbanda et al., 1998a, b). Each of these factors in the apoptotic cascade represents a potential target for blocking the effects of cytotoxic treatments on the ovary. However, a potential concern is that blocking apoptosis in DNA damaged oocytes could allow survival of cells that are beyond the repair capabilities of the DNA repair molecules and would normally have been directed down the apoptotic pathway. Fertilization of these genetically compromised oocytes could lead to an increased risk of fetal death or malformation.

SIRT1 and SIRT6 are additional factors involved in the regulation of cell death and survival. Recent data demonstrate that inducing overexpression of SIRT1 and SIRT6 via calorie restriction promotes cell survival by increasing cellular resistance to oxidative stress and apoptosis and promoting DNA repair (Cohen et al., 2004; Matsushita et al., 2005; Alcendor et al., 2007; Jeong et al., 2007; Mao et al., 2011). In the mouse ovary, calorie restriction was shown to up-regulate SIRT1
expression and protect the ovarian reserve against chemotoxic insult (Xiang et al., 2012). In light of the concern regarding the promotion of cell survival in genetically compromised oocytes, it is interesting to note that evidence suggests that SIRT1 acts primarily by enhancing cellular defense and repair mechanisms rather than simply by blocking apoptosis, and thus cells retain the ability to undergo apoptosis if the damage is beyond repair (Cohen et al., 2004).

**Chemotherapy induces dormant follicle activation**

A recent study proposed a novel theory of chemotherapy-induced destruction of dormant follicles, suggesting that in vivo chemotherapy triggers follicle activation and growth, causing burnout and depletion of the ovarian reserve (Kalich-Philosoph et al., 2013). Histology demonstrated both an absence of apoptosis in primordial follicles together with an initiation of follicle growth immediately following chemotherapy exposure, occurring simultaneously with large follicle apoptosis. The follicle activation was shown to be mediated by an up-regulation in the PI3K/PTEN/Akt signaling pathway (Fig. 3A and B), whose role in follicle quiescence has been well established by numerous knock-out mouse models (Adhikari and Liu, 2009) as well as by in vitro studies on human cortical tissue (Li et al., 2010; Kawamura et al., 2013). The PI3K/PTEN/Akt pathway is also believed to trigger follicle activation in vivo in human ovaries in response to mechanical disruption of the tissue and culture with an Akt stimulator (Kawamura et al., 2013). The route by which chemotherapy terminates follicle dormancy and induces activation of the PI3K/PTEN/Akt pathway may be via direct influence on the oocytes and granulosa cells of primordial follicles, or indirectly via chemotherapy-induced destruction of larger follicles (Roness, 2013). Destruction of large follicles removes the negative regulation of the primordial follicle pool thereby resulting in activation of the primordial follicles in an attempt to replace the dying cohort of growing follicles. This has been suggested as the mechanism behind primordial follicle activation and depletion seen following exposure to carcinogen and ovotoxicant 3-methylcholanthrene (Sobinoff et al., 2012), and is the underlying principal behind the clinical practice of using cytotoxic pretreatments to induce stem cell mobilization.

**Stromal effects of cytotoxic treatments**

Histological studies also point to indirect effects of chemotherapy via stromal cell damage (Marcello et al., 1990). Anti-neoplastic agents have been associated with a variety of heterogenous vascular complications (Doll et al., 1986). In vivo ultrasound monitoring of blood flow following doxorubicin administration has shown a significant acute reduction in ovarian blood volume and narrowing of the small vessels (Bar-Joseph et al., 2011). Examination of human ovarian tissue previously exposed in vivo to non-sterilizing doses of combined chemotherapy (Meirow et al., 2007) shows evidence of thickening and hyalinization of cortical stromal blood vessels, proliferation of small, non-mature, disorganized blood vessels in ovarian cortex or neovascularization, and subcapsular focal cortical fibrosis. These results are compatible with those of other studies that have shown that chemotherapy results in stromal fibrosis (Nicosia et al., 1985).

In cow and human ovaries primordial follicles are found predominately in the ovarian cortex, a vessel-poor region of the ovary (Herrmann and Spanel-Borowski, 1998; Motta et al., 2002). In contrast, the area surrounding the medulla, containing pre-antral and antral follicles, is a well vascularized zone (Herrmann and Spanel-Borowski, 1998). Since blood supply to the ovary is an end artery system (Reeves, 1971), chemotherapy-induced blood vessel obstruction causes localized ischemia damage causing the loss of primordial follicles in that area (Meirow et al., 2007). Vascular damage is therefore an indirect mechanism by which chemotherapy reduces the primordial follicle reserve.

**Prevention/reduction of cytotoxic ovarian damage**

**Currently available options**

The two established methods of fertility preservation are embryo and oocyte cryopreservation (Pfeifer et al., 2013), while ovarian tissue cryopreservation is still considered experimental. Each method carries limitations that restrict their usefulness in fertility preservation. Embryo cryopreservation requires the patient to have a male partner or willingness to use donor sperm, as well as the ability to delay treatment for between 2 and 6 weeks to allow for stimulation and oocyte retrieval. Oocyte cryopreservation does not yet have the success rates of embryo cryopreservation, although improvements in technique have increased efficacy, and it also requires time for stimulation and retrieval. If cancer treatment cannot be delayed, some centers allow oocyte retrieval for embryo or oocyte cryopreservation soon after completing an initial round of chemotherapy (Brown et al., 1996); however in many cases there is no response to stimulation and no oocytes can be retrieved. In addition, retrieval at this stage engenders the additional risk of creating pregnancies from oocytes exposed to cytotoxic treatments during growth. The possibility that these preserved oocytes or embryos carry an increased teratogenic risk is a significant concern (Chung et al., 2013). Another key limitation of both embryo and oocyte cryopreservation is that they cannot be performed in prepubertal patients.

Ovarian tissue cryopreservation does not carry these same limitations but is still classified as experimental, and carries the additional risk of returning cancer cells to a ‘cured’ patient. All of these methods require invasive surgeries pre-chemotherapy, and expensive and risky assisted reproductive treatments when the patient is ready to conceive. In addition, the first two options are not available at all to prepubertal patients (Chung et al., 2013).

There are a number of criteria that could define a more effective method of preserving fertility in patients undergoing cytotoxic treatments. Such a method would be one that is suitable for patients of all ages and life stages, does not carry additional health risks, does not interfere with their current treatments and does not require subsequent invasive treatments to enable realization of their fertility in the future. This definition describes what has been termed ‘fertoprotective adjuvant therapy’ (Woodruff, 2009), a potential attenuating agent which can prevent the loss of the ovarian follicle reserve and thus preserve patient fertility.

While there are many advantages to protective agents, there are some potential risks that need to be considered (Table II). A primary concern should be the impact of any potential agent on the cancer treatment. If the agent interferes with the efficacy of treatment, it cannot be considered for use. An additional concern lies with agents which prevent follicle loss by interfering with cell death mechanisms. An agent which prevents the death of oocytes in the face of cytotoxic treatments may be enabling the continued existence of cells carrying significant genetic damage, and could therefore lead to the risk of fetal malformations and death. Any
proposed attenuating agent must be assessed in light of these risks before it can be considered for clinical use.

**Timeframe for preventative treatment**

Preventing the destruction of the ovarian reserve caused by cytotoxic treatments would require concomitant or prior administration of an attenuating agent at the time of treatment. A recent study on the absorption and distribution of DXR in the ovary showed that the drug causes measurable DNA damage in the stromal cells of the cortex within 2 h of administration, and within 4 h in the granulosa cells of the follicles (Roti Roti et al., 2012). *In vivo* imaging shows that already 3 min after DXR injection there is a significant decrease in ovarian blood volume (Bar-Joseph et al., 2011). This indicates that the window for preventing...
chemo-induced ovarian toxicity is very small, and that attenuating agents would likely have to be administered prior to, as well as, concurrently with chemotherapy treatments. Certain types of protective agents, such as hormonal suppressants, by nature of their method of action, would need to be administered for a period of weeks in order to bring the ovary to a new homeostasis prior to treatment.

**Potential agents for fertility preservation**
A summary of the agents with potential for prevention of cytotoxic-induced ovarian damage is presented in Table III.

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**Table II** Attenuating agents for fertility preservation: advantages, disadvantages and risks to consider.

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<th>Advantages</th>
<th>Disadvantages</th>
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</tr>
</thead>
<tbody>
<tr>
<td>• Suitable for patients of all ages</td>
<td>• Must be administered prior to and during treatment</td>
<td>• May interfere with cancer treatment</td>
</tr>
<tr>
<td>• Does not require sperm donor</td>
<td>• Some agents require local administration into the ovarian bursa (such as S1P)</td>
<td>• May result in survival of damaged oocytes</td>
</tr>
<tr>
<td>• Reduces need for subsequent invasive procedures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Low risk to health</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Can be used in conjunction with other fertility preservation measures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Cost effective</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SIP, sphingosine-1-phosphate.

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**Table III** Potential agents for prevention of cytotoxic-induced ovarian damage.

<table>
<thead>
<tr>
<th>Protective agent</th>
<th>Mechanism of action on ovary</th>
<th>Studies demonstrating protective effect in vivo</th>
<th>Studies demonstrating no effect</th>
<th>Interactions with cytotoxic treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalidomide</td>
<td>Unclear: Inhibition of angiogenic factors, suppression of pituitary-gonadal axis</td>
<td>Rodent: Ochalski et al. (2011)</td>
<td></td>
<td>Antitumor effects</td>
</tr>
<tr>
<td>AS101</td>
<td>Modulation of PI3K/PTEN/Akt follicle activation pathway</td>
<td>Rodent: Kalich-Philosoph et al. (2013)</td>
<td></td>
<td>Does not interfere with and may have additive/synergistic interaction with treatment drug.</td>
</tr>
</tbody>
</table>

* Demeestere et al. (2013) reported no change in POF incidence but higher AMH values in GnRH-a treated patients.

bThese studies examined radiation not chemotherapy.

G-CSF, granulocyte-colony stimulating factor.
GnRH analogs: suppression of the pituitary-gonadal axis

While suppression of the pituitary-gonadal axis via GnRH analog administration has resulted in a reduction in primordial follicle loss following chemotherapy treatment in rhesus macaques (Ataya et al., 1995) and mice (Meirov et al., 2004; Kishk and Mohammed Ali, 2013; Li et al., 2013), in humans the results of clinical trials are contradictory. A number of recent meta-analyses (Ben-Aharon et al., 2010; Kim et al., 2010; Yang et al., 2013), as well as the Cochrane Review of 2011 (Chen et al., 2011), concluded that GnRH analog co-treatment during chemotherapy does convey some measure of ovarian protection ovarian, but that since the data in most studies reviewed were non-randomized, ‘well-designed, large, prospective, randomized, controlled trials’ are necessary to confirm the protective effect of GnRH analogs. Several such prospective, randomized, controlled trials have been recently completed with conflicting results (Table IV). A large study (Del Mastro et al., 2011) assessed 281 women with breast cancer who received chemotherapy either alone or with GnRH analog, and found a significant decrease in POF 12 months post-treatment in women receiving the GnRH analog (8 versus 25%). Two Egyptian trials of 78 and 100 women with breast cancer, respectively, (Badawy et al., 2009; Elgindy et al., 2013) had conflicting results. One study found that 69% of those who received adjuvant GnRH analog treatment during chemotherapy resumed normal ovarian activity compared with 26% of patients who did not (Badawy et al., 2009); however, the second study found no difference between the treatment groups in resumption of menstruation or hormonal or ultrasound markers of ovarian function 1 year post-treatment (Elgindy et al., 2013). Three smaller trials conducted on 60 (Gerber et al., 2011), 47 (Munster et al., 2012) and 63 (Sverrisdottir et al., 2009) women also found no significant change in POF incidence of co-treatment with GnRH analog up to 3 years post-chemotherapy. A trial conducted on 84 women with Hodgkin’s and non-Hodgkin’s lymphoma (Demeestere et al., 2013) found no difference in the incidence of POF 1 year post-chemotherapy (~20%); however, AMH levels were significantly higher in women who received cotreatment with GnRH analog (1.4 compared with 0.56 ng/ml). The inconsistency in the results of these trials is due both to the different chemotherapy protocols as well as the different outcome definitions employed. While POF is an all or nothing determinant, the impact of chemotherapy on fertility is very often a partial reduction in ovarian reserve, which leads to subfertility and early menopause; such outcomes require more subtle measurements of ovarian reserve and longer time points. A recent meta-analysis attempted to pool the results of these randomized trials to reach an overall conclusion (Del Mastro et al., 2013), and while they found an overall significant protective effect of GnRH analogs in young cancer patients, they noted that this effect was only evident in certain types of cancer, and underlined the large heterogeneity of POF incidence across the studies. More randomized trials with longer follow-up periods and assessments of ovarian reserve rather than ovarian function will be necessary to clarify the protective influence of GnRH analogs during chemotherapy.

It is difficult to explain how GnRH analog administration confers a degree of protection to follicle reserves, since human primordial follicles are not directly under gonadotrophin influence (Rabinovici and Jaffe, 1990; Hsueh et al., 2000). There have been several suggested mechanisms, including the possibility that GnRH analogs decrease ovarian perfusion thus decreasing exposure of the primordial follicles to the cytotoxic agents (Meirov et al., 2007), and that GnRH analogs may up-regulate anti-apoptotic molecules such as sphingosine-1-phosphate (Blumenfeld, 2007).

Sphingosine-1-phosphate: inhibition of apoptosis via the sphingomyelin pathway

Manipulations of apoptotic pathways, specifically the sphingomyelin pathway, shown to be responsible for the apoptosis of ovarian follicles, have also shown potential in preventing premature follicle loss. Sphingomyelin hydrolysis is an important apoptotic trigger and sphingosine-1-phosphate (S1P) has been identified as an inhibitor of ceramide-promoted cellular demise (Perez et al., 1997; Morita et al., 2000). In vivo treatment of human ovarian tissue xenografts in mice with S1P was shown to increase vascular density and angiogenesis, and reduce follicle apoptosis (Soleimani et al., 2011). Mouse oocytes derived from wild-type females treated with S1P show resistance to DXR-induced apoptosis (Morita et al., 2000). S1P pretreatment prior to chemotherapy is not conclusively effective, with reports showing both a protective effect in mice treated with Dacarbazine (Hancke et al., 2007) and no reduction in follicle loss in Cyclophosphamide-treated rats (Kaya et al.,

<table>
<thead>
<tr>
<th>Randomized trial</th>
<th>No. of patients</th>
<th>Diagnosis</th>
<th>Time post-treatment</th>
<th>POF incidence in GnRH treated women (%)</th>
<th>POF incidence in controls (%)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Badawy et al. (2009)</td>
<td>78</td>
<td>Breast cancer</td>
<td>8 months</td>
<td>11</td>
<td>67</td>
<td>Yes</td>
</tr>
<tr>
<td>Sverrisdottir et al. (2009)</td>
<td>63</td>
<td>Breast cancer</td>
<td>3 years</td>
<td>14</td>
<td>18</td>
<td>No</td>
</tr>
<tr>
<td>Del Mastro et al. (2011)</td>
<td>281</td>
<td>Breast cancer</td>
<td>12 months</td>
<td>8</td>
<td>25</td>
<td>Yes</td>
</tr>
<tr>
<td>Gerber et al. (2011)</td>
<td>60</td>
<td>Breast cancer</td>
<td>2 years</td>
<td>0</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>Munster et al. (2012)</td>
<td>47</td>
<td>Breast cancer</td>
<td>6 months</td>
<td>12</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Demeestere et al. (2013)</td>
<td>84</td>
<td>Hodgkin and non-Hodgkin’s lymphoma</td>
<td>12 months</td>
<td>20</td>
<td>19</td>
<td>No</td>
</tr>
<tr>
<td>Elgindy et al. (2013)</td>
<td>100</td>
<td>Breast cancer</td>
<td>12 months</td>
<td>20</td>
<td>16</td>
<td>No</td>
</tr>
</tbody>
</table>
There is stronger evidence to show a protective effect against radiation, with in vivo administration of S1P before radiation exposure resulting in a dose-dependent preservation of follicle numbers in mice, and a virtually complete preservation of both primordial and growing follicles when S1P is administered at high doses (Morita et al., 2000). In similar studies, S1P pretreatment was shown to reduce irradiation-induced primordial follicle depletion in rats (Kaya et al., 2008), primates (Zelinski et al., 2011) and xenografted human ovarian tissue (Zelinski et al., 2011).

One limitation of S1P is that it cannot be administered systemically and must be injected directly into the ovary. In addition, there are two important concerns that are raised regarding the use of attenuating agents which maintain primordial follicle levels by interfering with or blocking apoptotic pathways, such as S1P and imatinib (below). Firstly, these agents will need to be evaluated as to whether they will interfere with the therapeutic effects of chemotherapy drugs, although local administration alleviates this concern somewhat. Secondly, blocking apoptotic pathways may prevent the natural atresia of DNA damaged oocytes, resulting in the future fertilization of genetically compromised oocytes which should have undergone apoptosis. Offspring derived from female mice and macaques which received S1P treatment prior to radiation showed no significant abnormalities of any kind (Paris et al., 2002; Zelinski et al., 2011), but no similar studies have been conducted on chemotherapy. **Imatinib: inhibition of apoptosis via c-Abl kinase**

Imatinib is a competitive tyrosine-kinase inhibitor used in cancer treatment, which has been proposed as an agent to prevent primordial follicle loss caused by cisplatin (Gonfloni et al., 2009) based on its role as a c-Abl kinase inhibitor. Results from that study demonstrated that co-administration of imatinib in mice reduced primordial follicle loss and improved fertility and reproductive outcomes. However, a subsequent study contested these results, finding that imatinib did not protect primordial follicle oocytes from cisplatin-induced apoptosis or prevent loss of fertility in two independent strains of mice (Kerr et al., 2012). While the role of c-Abl in the induction of oocyte degeneration has been confirmed using another c-Abl inhibitor GNF-2 (Maiani et al., 2012), concerns remain regarding the potential effects of imatinib on oocyte integrity (Woodruff, 2009), and further study is required, including an assessment of oocyte DNA damage following treatment as well as compatibility with cancer treatment.

**Thalidomide: inhibition of angiogenic factors, gonadal suppression**

Thalidomide is a potent inhibitor of vascular endothelial growth factor (VEGF) and tumor necrosis factor α, and has significant ‘antitumor’ activity, both alone and as an adjunct to traditional chemotherapy (Teo, 2005). When administered to mice for a week prior to and post busulfan treatment (Ochalski et al., 2011), there were small but significant increases in ovarian function and follicle reserve in thalidomide-treated animals, but no difference in FSH levels between treatment groups. The authors postulate that thalidomide reduces blood flow to the ovaries via its effect on VEGF, thereby reducing the amount of chemotherapy that reaches the ovary, or alternatively, by inducing a temporary state of ovarian senescence (Dharia et al., 2004), similar to GnRH analogs, although the mechanism for this is unclear. With only a single study examining the protective effects of thalidomide on the ovary, there are insufficient data to draw any conclusions. Furthermore, in light of its effects on VEGF it will be important to determine whether thalidomide interferes with the efficacy of cancer treatment.

**Tamoxifen: potentiation of IGF-I**

Tamoxifen is an estrogen receptor antagonist used in adjuvant treatment of hormone sensitive cancers. Two rodent studies have proposed a protective effect when tamoxifen is given during chemo/radiotherapy. Ting et al. showed that in vivo treatment with tamoxifen significantly reduces follicle loss caused by chemotherapy, as well as improves fertility outcomes post-treatment (Ting and Petroff, 2010). In addition the study demonstrated that while in vitro DXR exposure increased oocyte fragmentation, the effect was reversed with tamoxifen. The mechanism of tamoxifen-induced protection against chemotherapy has not been investigated and is thought to relate either to its effects on the pituitary-gonadal axis, or to its role as an estrogen antagonist with associated anti-apoptotic and antioxidant effects (Nathan and Chaudhuri, 1998; Dubey et al., 1999).

Similarly, while whole body irradiation in rats significantly reduces primordial follicle numbers and AMH serum levels, and increases oxidative stress markers, co-treatment with tamoxifen can ameliorate all of these effects, and rescue fertility post-treatment (Mahran et al., 2013). This study demonstrated that tamoxifen treatments resulted in a significant increase in both transcription and translation of IGF-I, which has been shown to modulate gonadotrophin action in the ovary by augmenting granulosa cell FSHR expression and potentiating FSH action (Zhou et al., 1997), as well as exert antioxidant and cryoprotective effects (Armstrong and Webb, 1997; Baeza et al., 2010).

**Granulocyte colony-stimulating factor: promotion of neovascularization?**

Granulocyte colony-stimulating factor (G-CSF) stimulates the bone marrow to produce granulocytes and stem cells, and has been shown to promote neovascularization following ischemia. In mice, it has been shown to significantly reduce the destruction of primordial follicles caused by cyclophosphamide and busulphan, and also prevents damage to the microvessels, and reduces markers of DNA damage in oocytes of early growing follicles to control levels (Skaznik-Wiikel et al., 2013). An extended mating study further demonstrated that mice which received G-CSF at the time of treatment produced significantly more pups per litter than those that received chemotherapy only. The link between the effects of G-CSF on blood vessels and the protection of the ovarian reserve requires clarification. It is possible that by stimulating new blood vessel formation, G-CSF decreases chemotherapy-related blood vessel loss and the associated focal ischemia shown by Meirow et al. (Meirow et al., 2007) as one cause of chemotherapy-related follicle loss. While confirmation of these data and further examination of the mechanism behind the protective effect of G-CSF are still warranted, it claims certain advantages over some proposed attenuating agents as it is currently in use clinically in cancer patients for prevention of chemotherapy-induced neutropenia and has been shown not to reduce the efficacy of chemotherapeutic agents.
**AS101: inhibition of the PI3K/PTEN/Akt follicle activation pathway**

The immunomodulator AS101 is a non-toxic, tellurium-based compound that acts on the PI3K/PTEN/Akt pathway (Makarovsky et al., 2003; Sredni, 2012). AS101 has been shown to protect against chemotherapy-induced reproductive damage and reduce sperm DNA fragmentation in male mice without interfering with the effectiveness of cytotoxic treatments (Carmely et al., 2009). In female mice, AS101 has been shown to inhibit the activation and loss of the dormant primordial follicles by chemotherapy via the PI3K/PTEN/Akt pathway, as well as to reduce apoptosis in the granulosa cells of growing follicles (Fig. 3C) (Kalich-Philosoph et al., 2013). Co-treatment with AS101 protected the ovarian reserve in cyclophosphamide-treated mice both in the short and long term, and preserved fertility, with reproductive outcomes similar to untreated animals. The successful reproductive outcomes and lack of any increase in fetal malformations in the group which received cyclophosphamide with AS101 indicates that the functionality and genetic integrity of these ‘rescued’ oocytes are not compromised. This and previous studies have also demonstrated that AS101 does not interfere with the primary anti-neoplastic activity of cyclophosphamide in vivo, or with cyclophosphamide metabolites in vitro, but has additive and synergistic anti-cancer activity (Kalechman et al., 1991, 1993; Sredni et al., 1992).

AS101 can be administered either via oral or intravenous routes. The biological properties associated with AS101 are believed to stem from the ability of the tellurium atom to associate with some thiols, such as cell membrane cysteine residues, resulting in anti-apototic and anti-inflammatory activity. AS101 has also demonstrated direct inhibitory effects on PI3K/PTEN/Akt signaling pathway (Hayun et al., 2006), and inhibits specific integrins (Indenbaum et al., 2012); therefore it may be possible that AS101 down-regulates the PI3K/PTEN/Akt pathway via inhibition of integrins. Clinical studies will be needed to verify the ability of AS101 ability to prevent toxicant-induced ovarian damage in female cancer patients.

**Other potential methods of reducing chemotherapy-induced ovotoxicity**

Another direction that has been investigated to reduce the toxic side effects of chemotherapy in particular for use with solid tumors, is drug encapsulation with/without tumor targeting. A recent study examined the possibility that nano-encapsulation of Arsenic trioxide (As$_2$O$_3$) (used in the treatment of hematological malignancies) would increase its efficacy against solid tumors while simultaneously reducing its effects on the ovary (Ahn et al., 2013). The study demonstrated that nano-encapsulation of As$_2$O$_3$ resulted in lower peak plasma levels, limited its tissue distribution, and reduced its impact on ovarian and follicular function. While the study did not examine the impact of the encapsulated drug on ovarian reserve or function following treatment, the concept holds great potential and warrants additional investigation.

**Conclusion**

Advances in our understanding of the mechanisms and pathways involved in both cytotoxic ovarian damage and follicle growth and development have opened up new avenues for fertility preservation. In a parallel fashion, the study of potential ovo-protective agents has contributed in significant ways to our understanding of ovarian physiology and the pathways involved in cellular injury and repair. Different cytotoxic agents act in specific ways on the different cell populations within the ovary, providing numerous targets for potential attenuating agents. Interference with any one of these pathways of injury has the potential to reduce the impact of cytotoxic agents on the ovary. Most protective agents discussed here are in very preliminary stages of study and future advances in this area will depend on accurate evaluation of the efficacy of each potential agent. This requires that studies employ two concrete end-point criteria; the number of follicles remaining post-treatment, and reproductive outcomes. Further to proof of efficacy, there exists an equally important requirement to demonstrate that co-treatment with these agents does not interfere with the anti-cancer activity of the chemotherapy drugs, or produce genetically comprised embryos. Beyond this, experience with GnRH analogs has demonstrated the imperative for carefully designed prospective randomized clinical trials. Only once all these criteria are met, we can begin offering attenuating agents alongside existing fertility preservation options in the clinic.

**Authors’ roles**

H.R., L.K.-P. and D.M. reviewed the literature and wrote the manuscript.

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**Conflict of interest**

None declared.

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