THERAPY OF ENDOCRINE DISEASE

Novel protection and treatment strategies for chemotherapy-associated ovarian damage

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Abstract

Fertility and ovarian protection against chemotherapy-associated ovarian damage has formed a new field called oncofertility, which is driven by the pursuit of fertility protection as well as good life quality for numerous female cancer survivors. However, the choice of fertility and ovarian protection method is a difficult problem during chemotherapy and there is no uniform guideline at present. To alleviate ovarian toxicity caused by anticancer drugs, effective methods combined with an individualized treatment plan that integrates an optimal strategy for preserving and restoring reproductive function should be offered from well-established to experimental stages before, during, and after chemotherapy. Although embryo, oocyte, and ovarian tissue cryopreservation are the major methods that have been proven effective and feasible for fertility protection, they are also subject to many limitations. Therefore, this paper mainly discusses the future potential methods and corresponding mechanisms for fertility protection in chemotherapy-associated ovarian damage.

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Introduction

Cancer is a problem that affects people all over the world. About 5.8% of female patients with cancer are younger than 50 years, and there is a rising trend in children and young women (1). Chemotherapy is an important means of cancer treatment, and majority of female patients with cancer experience adverse effects from chemotherapy every year. The gonadal toxicity of chemotherapy may result in premature ovarian failure (POF) or premature ovarian insufficiency (POI), which can lead to loss of fertility and damaged ovarian function. With the improvement in the survival rate of young women with malignant tumors, female cancer survivors have begun to focus on their fertility and endocrine function, which have important roles in women's psychological health, family well-being, and social stability.

The first pharmaceutical treatment for protecting the ovary from chemotherapy-associated damage was proposed by Romona in 1981 with the use of oral contraceptives. Since then, an increasing number of different types of approaches for protection from chemotherapy-induced ovarian damage have been reported. In this review, we briefly describe the currently available approaches and discuss mainly the future potential strategies that may be available for protecting ovarian function in patients undergoing chemotherapy.

Chemotherapy-associated ovarian damage

The most common cancers that occur in girls are acute lymphocytic leukemia, acute myeloid leukemia and

lymphoma. Girls with hematological malignancies are often treated with aggressive gonadotoxic anticancer regimens that include alkylating chemotherapy drug. Therefore, the risk of gonadotoxicity and subsequent POI/POF, and even fertility loss, is very high. In addition, breast cancer is the most common cancer in women of reproductive age, with more than 10% of new cases diagnosed in women younger than 40 years (2). Similarly, chemotherapy regimens that include alkylating agents with particular gonadotoxicity are often used. About 50% of young women became infertile or have reduced reproductive function after breast cancer chemotherapy (3). Therefore, it is important to pay attention to chemotherapy-associated ovarian damage.

The risk of developing POF or POI after chemotherapy is dependent on various factors. The first factor is the type of chemotherapy drug. Alkylating agents such as cyclophosphamide (CTX) and busulfan are more toxic to the ovary than any other agents. Within the ovarian follicles, both oocytes and granulosa cells are vulnerable to chemotherapy damage; Table 1 lists each class of chemotherapeutic drugs in the order of estimated

high risk to low risk of gonadotoxicity. In addition, chemotherapeutic dosage is another important dose-dependent factor in ovarian function. Dynes's group showed in a mouse model that, compared to the maximum nonlethal dose of CTX treatment, low-dose metronomic delivery of CTX is less detrimental to granulosa cell viability, ovarian function, and fertility (4). Furthermore, the patient's age is also a key factor. As aged group women have less primordial follicle reserve compared with young women, they are more likely to experience ovarian failure during or immediately after treatment. Therefore, older women tend to develop POI or POF after chemotherapy.

How do chemotherapy drugs damage the ovary?

Based on the targeted cell-cycle phases, antitumor agents are classified into cell cycle-specific and cell cycle-nonspecific; the former acts only on cells at a specific phase and the latter targets cells in all phases. As most of the primordial follicles remain quiescent in the ovary, they are more sensitive to cell cycle-nonspecific drugs. However, due to the active proliferation of granulosa cells

Table 1 The commonly used antitumor drugs of gonadotoxicity.

Gonadotoxicity/drugs	Species	Chemotherapy regimen [†]	Affected cell type	Affected follicle class	References
High risk					
Cyclophosphamide	Human*	75 mg/kg	Oocytes	Primordial	(39)
Cyclophosphamide	Mouse	120 mg/kg	GL cells	Growing follicles	(68)
Cyclophosphamide	Rat	200 mg/kg	GL cells	All follicular stages	(69)
Cyclophosphamide	Human**	200 mg/kg	Oocytes, stroma, GL cells	Primordial	(70)
Cyclophosphamide	Human**	200 mg/kg	Oocytes, preGL cells	Primordial	(71)
Cyclophosphamide	Mouse	300 mg/kg	Oocytes, GL cells	All follicular stages	(72)
Busulfan	Mouse	36 mg/kg		Primordial	(73)
Busulfan	Rat	0.1–1.5 mg/kg/day for 2 or 4 weeks		Primordial, primary	(74)
Procarbazine	Human			Antral follicles	(75)
Medium risk					
Doxorubicin	Mouse	7.5 or 10 mg/kg	GL cells	Secondary/pre-antral	(76)
Doxorubicin	Mouse	10 mg/kg	Oocytes, GL cells	All follicular stages	(77)
Carboplatin	Mouse	80 mg/kg on days 1, 7		All follicular stages	(20)
Cisplatin	Rat	5 mg/kg		Primordial, primary	(45)
Doxorubicin/Cisplatin	Mouse [‡]	DOX: 0.4 µM or 10 µM; Cisplatin: 10 µM	Oocytes	Primordial	(28)
Doxorubicin/Cisplatin	Mouse [‡]	Cisplatin: 0.1–5 µg/mL; DOX: 0.01–0.2 µg/mL	Oocytes, GL cells	Primordial	(32)
Low risk					
Paclitaxel	Mouse	2.5, 5.0, or 7.5 mg/kg		Primordial	(78)
Paclitaxel	Rat	7.5 mg/kg		Primordial	(79)
Paclitaxel	Rat	7.5 mg/kg		Primordial, primary	(80)
Methotrexate	Mouse	5 g/m ²		Primordial	(81)
Methotrexate	Rat	50 mg/m ² ; Multiple dose (1 mg/kg) on days 1, 3, 5, 7		All follicular stages	(82)
Vincristine	Mouse	1 mg/kg on days 1, 4, 8		Growing follicles	(83)
Gemcitabine	Rat	200 mg/kg	GL cells	Pre-antral/antral	(69)

GL, granulosa. *Human ovarian xenografting; **Human fetal ovarian xenografting; [†]provided as a single dose or as specified; [‡]*in vitro*.

in growing follicles, these follicles can be damaged by all kinds of antitumor agents. In general, chemotherapy drugs induce DNA damage, and persistent unrepaired DNA damage mainly activates the apoptosis of granulosa cells and oocytes. In addition, chemotherapy drugs also cause increased damage to blood vessels in the ovarian medulla by promoting the immune inflammation response. Excessive oxidative stress triggers apoptosis or induces the activation of dormant primordial follicles and also damages the ovarian microvessel network such as via vessel sclerosis and obliteration, leading to ischemia and nutrient deprivation of the follicles. A summary of ovarian damage caused by chemotherapy is depicted in Fig. 1.

Choice of chemotherapy regimen

As an adjuvant therapy, chemotherapy plays an important role in cancer treatment. The chemotherapy regimen and dosage are important factors that determine the degree of chemotherapy damage to ovarian function. When choosing a chemotherapy regimen, clinicians must consider the pathological type of the tumor, the sensitivity of tumor cells to chemotherapy drugs, and the antitumor

mechanism of the chemotherapy drugs. Therefore, under the premise of ensuring antitumor efficacy, chemotherapy drugs with less ovarian toxicity should be used as much as possible.

With the progress in medical scientific research and the increase in clinical trials, researchers constantly optimize the selection of chemotherapy regimens to achieve the best survival time and minimum adverse reactions. For example, Kerbrat's group conducted a randomized phase III trial comparing six cycles of 5-fluorouracil, epirubicin, and CTX (FEC) to four cycles of adriamycin and CTX (AC) in patients with breast cancer, and showed that there was no significance in both disease-free survival and overall survival between the FEC and AC regimens, while reproductive toxicity was more severe in the FEC regimen (5). These results indicate that four cycles of the AC regimen for breast cancer treatment inflict less damage on ovarian function.

In recent years, significant progress has been made in cancer treatment, especially in hematologic malignancies including Hodgkin lymphoma, non-Hodgkin lymphoma, and acute lymphoblastic leukemia. Now, increasing attention has focused on treatment-related complications, and determining how these complications can be reduced while ensuring therapeutic

Figure 1

The mechanism of ovarian damage caused by chemotherapy. Left: Different gonadotoxic drugs from high to low risk; middle: three pathways (DNA damage, immune inflammation, oxidative stress) coupled with their action factors of chemotherapy-associated ovarian damage; right: pathology results of ovarian follicle, ovarian stroma, and blood vessel after chemotherapy. A full color version of this figure is available at <https://doi.org/10.1530/EJE-20-1178>.

efficacy has become a research hotspot. In 2018, to compare the ovarian toxicity of different chemotherapy regimens for treating advanced Hodgkin lymphoma, Anderson's group compared the ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) and BEACOPP (bleomycin, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone, gemcitabine) regimens, and showed that women treated with BEACOPP had high potential for gonadotoxicity compared with those treated with ABVD (6). McLaughlin's group compared the density, morphology, and *in vitro* developmental potential of human ovarian follicles treated with the ABVD and OEPA-COPDAC (combined vincristine, etoposide, prednisone, doxorubicin (OEPA) and cyclophosphamide, vincristine, prednisone, dacarbazine (COPDAC)) chemotherapeutic regimens. To their surprise, the density of non-growing follicles (NGFs) was increased following ABVD, with higher NGFs per mm³ (230 ± 17) (mean $\pm$ S.E.M.) than untreated (110 ± 54), OEPA-COPDAC treated (50 ± 27), and obstetric (20 ± 4) tissue ($P < 0.01$) (7). The above studies demonstrate that ABVD regimens may be a good choice for preserving ovarian reserve in patients with Hodgkin lymphoma.

In addition, nanotechnology is a remarkable example of human achievement, and nanodrugs are one of the applications of nanotechnology for improving human health through better diagnoses and treatments. In particular, nanotechnology shows great potential for treating cancers, where nanoparticles are used as drug delivery carriers (8). The nanocarrier-based delivery system of anticancer drugs has been noteworthy in recent years because of its potential for increasing the efficacy of the drugs and decreasing adverse effects. Based on the difference between normal tissues and tumor tissues, inorganic nanocarriers can ensure that drugs selectively accumulate toward tumor tissues. For example, cisplatin-conjugated glyconanoparticles showed superior anticancer efficacy than free cisplatin as they could target cancer cells better, showing higher cytotoxicity and high usage in cancer cells, with the results being decreased plasma level and toxicity of these drugs. In addition, a study on mouse models of lymphoma has shown that a nanomaterial-coated arsenic trioxide can inhibit tumor proliferation and reduce the damage of drugs to ovarian reserve effectively (9). Therefore, nanodrugs decrease the toxicity related to the basic mechanism of the drug action and show low levels of nonspecific toxicity.

Although the ranking of chemotherapeutic drugs from high to low risk of gonadotoxicity is known, most cancer treatments involve combination chemotherapy,

requiring two or more drugs, while few studies compare the gonadotoxicity among different combination chemotherapy approaches in the clinic. Therefore, more clinical trial studies are needed and clinical oncologists should pay more attention to ovarian function during chemotherapy.

Currently available options for ovarian protection in chemotherapy

Embryo and oocyte cryopreservation

Embryo cryopreservation has been the gold-standard option for female fertility preservation for decades. Since the birth of the first child following *in vitro* fertilization embryo transfer (IVF-ET) in 1978, the IVF-ET technology has developed for more than 40 years. Recently, it was reported that frozen single blastocyst transfer resulted in a higher singleton live birth rate than fresh single blastocyst transfer, and frozen embryo transfer resulted in a lower risk of ovarian hyperstimulation syndrome (10). The results of that study can eliminate the concerns of patients with cancer about the poor quality of frozen embryos.

The American Society for Reproductive Medicine included oocyte cryopreservation as a standard therapy for fertility preservation in 2012; since then, it has been adopted as a therapy worldwide. Comparisons of cryopreserved and fresh oocytes for IVF and intracytoplasmic sperm injection have shown similar fertilization and pregnancy rates for fresh and cryopreserved oocytes with IVF (11, 12). Importantly, it has been indicated that cryopreserved oocytes have no increased risk of congenital anomalies compared with the general population or with those conceived after fresh IVF (13).

Although embryo and oocyte cryopreservation are well developed, there are some inherent deficiencies. The process of obtaining oocytes by ovulation induction will delay the treatment of tumors. In addition, it may increase the risk of invasion and metastasis with sex hormone-dependent tumors when ovulation is stimulated with exogenous hormones (14). Meanwhile, embryo or oocyte cryopreservation cannot be conducted in prepubertal girls.

Ovarian tissue cryopreservation

Due to the improvement in medical technology, ovarian tissue cryopreservation and transplantation (OTCT) have gradually been established from the experimental stage.

In 2005, the first successful live birth was produced by IVF-ET after orthotopic transplantation of cryopreserved thawed ovarian tissues. OTCT has resulted in the restoration of endocrine function (ranging from 6 months to 10 years) in around 93% of cases and resulted in more than 130 live births to date (15). The advantages of ovarian tissue transplantation include eliminating the need for ovarian stimulation or exogenous hormones and the immediate initiation of cancer therapy. At present, auto-transplantation is the only option which is able to re-establish ovarian function from cryopreserved ovarian tissue in cancer survivors.

Despite these successful results, most countries still consider OTCT as an experimental procedure due to its complex technology. In addition, there should be concern about its safety in patients with oncological recurrence during autologous transplantation, especially in leukemia survivors. At present, the risk of recurrent oncological disease due to the reintroduction of cancer cells via the auto-transplantation of cryopreserved ovarian tissue is unknown. In the future, more studies assessing the safety of autologous ovarian transplantation in patients with cancer should be reported.

Gonadotrophin-releasing hormone analogs

One of the suggested strategies for preserving fertility is ovarian suppression by downregulating the hypothalamic–pituitary–ovarian axis with gonadotropin-releasing hormone analogs (GnRHa) before and during chemotherapy. This creates a pseudo-menopausal state with decreased ovarian function and maintains the ovary in a quiescent state. The primary mechanism of action of GnRHa is to suppress the gonadotropin levels to simulate the prepubertal hormonal milieu and subsequently prevent primordial follicle activation; therefore, it decreases the number of growing follicles, which are more vulnerable to chemotherapy. In addition, GnRHa can reduce the blood supply to the ovary, decrease drug accumulation, and hence may partly reduce the accumulation of chemotherapy drugs in the ovaries.

Some studies have suggested that administering GnRHa during chemotherapy protects ovarian function, whereas other studies did not show this effect. In Yang's study, five randomized controlled trials (RCTs) composed of 528 patients were included in a meta-analysis; the results showed that concurrent administration of GnRHa during chemotherapy treatment of breast cancer in premenopausal women appears to protect against chemotherapy-related POF in the first year after treatment

but appears to have no effect on resumed menses or spontaneous pregnancy rates (16). In a recent study, a total of 12 clinical RCTs were included in a meta-analysis (17), and the study revealed that GnRHa may significantly improve the menstrual function recovery rate in patients who undergo chemotherapy and may reduce the rate of POF. In 2018, when discussing GnRHa in fertility preservation, seven RCTs, four systematic reviews, and seven guidelines provided the evidence base for GnRHa in fertility preservation, and the comprehensive results showed that only a few studies recommend using GnRHa for fertility preservation in premenopausal patients with breast cancer, while the other studies do not recommend it (18).

Therefore, considering the current state of the evidence discussed previously, GnRHa should be used with caution as a valid fertility preservation method at present. The RCTs had overall heterogeneity, and their differences in disease, chemotherapy, follow-up time, and POF definition may have affected the results. To prove whether GnRHa can be used for protecting ovarian function, the most important work is to explore the fundamental research, such as whether GnRHa can influence or disrupt the efficacy of each kind of chemotherapy drug as well as the protective effect for each follicle stage.

Novel strategy options for ovarian protection and treatment in chemotherapy

Protective agents

As the overactivation and apoptosis of primordial follicle play important roles in chemotherapy-associated ovarian damage, substances working against the factors triggering follicle activation or substances blocking the apoptotic pathways have been proposed as potential candidates for preventing chemotherapy-associated ovarian damage. These substances are reviewed in this section and are summarized in Table 2.

Anti-Müllerian hormone

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance, is specifically produced by granulosa cells from the primary follicles to early antral follicle. Several experimental models show that AMH is implicated in the inhibition of the recruitment of primordial follicles. The ovaries of AMH^{-/-} knockout mice have decreased

Table 2 Protective agents for ovarian protection in chemotherapy. The species tested was mice except where otherwise indicated.

Protective agent	Drugs	Function	Molecular mechanism	References
AMH	Cyclophosphamide, Carboplatin, Doxorubicin Cyclophosphamide	Inhibits PMF activation Inhibits PMF activation	Acts directly on PMF FOXO3A phosphorylation; autophagy (LC3,P62)	(20) (21)
ATR inhibitors ETP46464, AZD6738 CHEK1/2 inhibitors CK2II BML-277 CHIR-124, MK-8776 CK1 inhibitors PF670462 c-Abl kinase inhibitors Imatinib	Cyclophosphamide Cyclophosphamide Cyclophosphamide Doxorubicin Cisplatin Doxorubicin	Inhibits PMF apoptosis Inhibits PMF apoptosis Inhibits PMF apoptosis Inhibits PMF apoptosis Inhibits PMF apoptosis Inhibits PMF apoptosis	ATR-CHEK1-TAp63 α -cPARP CHEK1/2-TAp63 α -cPARP Acts on the activation of TAp63 α CHEK1-TAp63 α Acts on the activation of TAp63 α	(29) (29) (28) (30) (28)
Melatonin Resveratrol miR-10a/miR-146 miR-21 let-7a	Cisplatin Cisplatin Cisplatin Epirubicin Cisplatin Cyclophosphamide, Busulfan Cyclophosphamide, Busulfan Cyclophosphamide 4-Hydroxyperoxy cyclophosphamide	Inhibits PMF apoptosis Inhibits PMF apoptosis Inhibits PMF activation Inhibits the loss of follicles Inhibits follicle apoptosis* Inhibits oogonal stem cell apoptosis Inhibits follicle atretic Inhibits granulosa* cells atretic Inhibits PMF apoptosis	c-Abl-TAp63/TAp73 c-Abl-TAp63 PTEN/AKT/FOXO3a Reduces ROS-induced ERS Prevents oxidative damage Nrf2, SOD2 p53-Bim-Casp9 PTEN and PDCD4 FASL and TLR5	(30) (28) (42) (84) (45) (43) (47) (48) (49)

*Investigated in rat.

primordial follicles and increased growing follicles compared with WT ovaries, which showed relatively early depletion of their stock of primordial follicles. In addition, *in vitro* experiments on human, bovine, and rodent ovaries have revealed improved transition from primordial to growing follicle in the absence of AMH, whereas this activation was blocked when AMH was added to the culture medium (19). Besides, AMH inhibits follicle-stimulating hormone (FSH) effects and is involved in the initial recruitment of primordial follicles. Kano *et al.* reported that supraphysiological doses of AMH could decrease the loss of primordial follicles induced by CTX, cisplatin, and doxorubicin chemotherapies in mice (20). In this study, AMH was administered the day before chemotherapy, and ovarian function was assessed 2 weeks after chemotherapy. In addition, a recent study evaluated the ovarian protective effects of AMH on pubertal mice treated with CTX (21). In that study, the authors confirmed that AMH plays an important role in the recruitment of primordial follicles. At 15 weeks after the end of treatment, the cumulative number of pups and the number of ovulated eggs after ovarian simulation were greater in mice that had received both treatments as compared with that of mice that had received CTX alone. In summary, the above results suggest that AMH could act as a major factor regulating primordial follicle recruitment.

Another important function of AMH in the ovary may be its anti-apoptotic effect. AMH knockout mice had accelerated follicular atresia compared to WT mice (22). In addition, in women undergoing IVF cycles, there is a negative correlation between granulosa cell apoptosis and follicular AMH levels (23). Additionally, women with polycystic ovarian syndrome (PCOS; known to have high AMH levels) have significantly lower granulosa cell apoptotic rates compared to women with normal AMH levels (24). These data support the hypothesis that AMH has anti-apoptotic effects on granulosa cells. Therefore, we may hypothesize that supplementation of AMH may prevent the chemotherapy-induced apoptosis of growing follicles.

AMH may be a particularly interesting new agent for protecting the ovarian reserve and subsequent fertility, as it acts as a targeted therapy. Recently, AMH has also emerged as a neuroactive peptide in regulating neuronal viability and activity in various brain regions (25). The evidence suggest that AMH potently activates the GnRH neuron to increase GnRH-dependent luteinizing hormone (LH) pulsatility and secretion but not between AMH and the FSH response dose (25). However, the GnRH activation

Figure 2

A detailed depiction of anti-DNA damage molecules for the TAp63 α pathway. The three classical ATM-CHEK2, ATR-CHEK1 and CK1 pathways activate TAp63 α via phosphorylation in response to DNA damage; the downstream targets of TAp63 α , mainly via two proapoptotic proteins PUMA and NOXA, activate the expression of BAX and BAK, which are essential for the initiation of apoptosis signaling. A full color version of this figure is available at <https://doi.org/10.1530/EJE-20-1178>.

induced by AMH is contrary to the effects of using GnRHa to protect against chemotherapy-associated ovarian damage in the clinic. Therefore, more fundamental and clinical research are needed to prove the feasibility of AMH for protecting ovarian function. If AMH is proven to be effective clinically, it could be a good agent for fertility protection during chemotherapy.

Anti-DNA damage molecules

The p53 protein family with its three members, that is p53, p63, and p73, play a vital role in the surveillance of genetic and cellular stability. The most ancient function of this family is probably the maintenance of genetic quality in germ cells, as both lower organisms and human primordial oocytes contain a high concentration of TAp63 α protein. However, TAp63 α protein is a double-edged sword, and its high expression in oocytes renders them sensitive to DNA damage. When cellular DNA damage occurs, the

DNA damage checkpoint kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) are activated through phosphorylation, which then activates the phosphorylation of the CHEK1/CHEK2 kinases and their downstream apoptotic pathways, such as the TAp63 α pathway (26). Besides, c-Abl phosphorylates TAp63 α at specific tyrosine residues induces TAp63 α -dependent activation of the proapoptotic pathway (27). A recent study by Tuppi *et al.* also identified CK1 as an essential kinase that activates TAp63 α (28). PUMA and NOXA, two downstream targeted molecules of TAp63 α , initiate apoptosis by activating the multi-BH domain proapoptotic BCL-2 family members, BAX and BAK, which are essential for the initiation of apoptosis signaling. Therefore, the TAp63 α pathway plays an important role in chemotherapy-associated ovarian damage (Fig. 2).

ATR, CHEK, and CK1, which have specific inhibitors, are the kinases acting upstream of TAp63 α ; therefore, targeting these kinases offers promise for protecting oocytes against chemotherapeutic drugs. Several studies have indicated that ATR, CHEK, or CK1 inhibitors prevent the apoptosis of primordial follicles by inhibiting the TAp63 α pathway (Table 2). Luan showed that treatment with ATR and CHEK2 inhibitors blocked 4-hydroperoxycyclophosphamide-induced oocyte DNA damage *in vitro* (29). In addition, So-Youn *et al.* demonstrated that CHEK2 inhibitors used 2 h before the administration of 0.1 Gy X-ray or 5 mg/kg cisplatin could preserve ovarian function (30). Tuppi *et al.* also determined that CHEK2 and CK1 inhibitors reduced the oocyte DNA damage induced by cisplatin *in vivo* (28). Some of the previously mentioned inhibitors have already been used in clinical trials as anticancer therapeutics. Therefore, CHEK1/CHEK2 or CK1 inhibitors have the potential feasibility of adjuvant therapies for protecting ovarian function without affecting the anticancer efficacy of chemotherapy.

In addition, PUMA and NOXA are effective downstream targets of the TAp63 α pathway. Female mice deficient in PUMA or NOXA were substantially protected from irradiation- and chemotherapy-induced primordial follicle apoptosis and remained fertile (31). The health assessment of the offspring showed that their health or genotype was not compromised by either of the previously mentioned treatments. Therefore, specifically regulating the expression of PUMA in the ovary without affecting the treatment of malignant tumors is a good strategy for ovarian protection.

Further, imatinib, an inhibitor of the oncogenic BCR-Abl tyrosine kinase enzyme, prevented the activation of c-Abl/TAp63 α in mouse oocytes and thereby protected them from cisplatin-induced apoptosis. In 2009, Stefania *et al.* showed that treatment with the c-Abl kinase inhibitor imatinib during chemotherapy could inhibit the cisplatin-induced apoptosis of primordial follicles in mice (28). Subsequently, Morgan *et al.* reported that imatinib also protected follicles against damage induced by cisplatin but not against doxorubicin in mouse ovaries *in vitro* (32). However, one study demonstrated that imatinib did not protect the oocytes of primordial follicles from cisplatin-induced apoptosis in two strains of mice (33). Further, Zamah demonstrated that imatinib itself could compromise ovarian function during treatment in mice (34). Recently, it has been reported that imatinib treatment resulted in the apoptosis of granulosa cells and oocytes in human ovaries, and similar toxic effects were also observed when the ovarian tissues were transplanted into xenograft animal models (35). Therefore, imatinib as a treatment for fertility protection is controversial, and further study is required, including an assessment of its cytotoxic effects on oocytes as well as the protective effects on ovarian function during cancer treatment.

In summary, the anti-DNA damage molecules, such as the inhibitors of PUMA, CHEK1, CHEK2, and CK1, are promising drugs for ovarian protection. However, more studies should be conducted to elucidate the question of whether the mechanism also holds true for treatment in humans. In addition, further assessment of offspring health is warranted. Childhood cancer survivors are exposed to high doses of potent mutagens in the form of chemotherapy and radiation therapy that might affect human germ cells and cause potential transmissibility of germline damage to offspring. To assess the reproductive risk of chemotherapy, the genetic integrity of the offspring must be quantitatively assessed by whole-genome or exome sequencing. As these procedures are not yet widely used in the clinic, future research should focus on the safety aspects of these inhibitors.

Anti-apoptosis agents

Sphingosine-1-phosphate (S1P), which is synthesized by sphingosine kinase from sphingosine, is a downstream anti-apoptotic metabolite of ceramide. Moreover, specifically in female gonads, these sphingolipid-based signaling events have been identified as key mediators in the regulation of oocyte apoptosis. These findings raise the possibility that oocyte damage caused by chemotherapy

can be partly prevented by manipulating the anti-apoptosis molecule, S1P. One study showed that mature oocytes were protected from doxorubicin-induced cell death by the addition of S1P into the culture medium *in vitro* (36). Hancke also showed that local ovarian injection of S1P protected ovarian follicles from chemotherapy-induced cell death, thereby preserving fertility in mice (37). In addition, S1P and its analog (FTY720), given for 1 week before irradiation, indicated a protective effect on ovarian function against radiotherapy damage in female rhesus macaques (38). Subsequently, in a human ovarian study, S1P blocked primordial follicle death induced by both CTX and doxorubicin in a human ovarian cortical xenograft model (39). Therefore, S1P and its analogs hold promise for preserving fertility in patients undergoing chemotherapy.

However, there are two concerns about the use of S1P with chemotherapy. One issue is the impact of S1P on cancer cells. Although one study showed that S1P treatment did not reduce the effectiveness of chemotherapy on S180 sarcoma cells *in vitro* (40), no similar data were available in a tumor xenograft model. The other problem is the integrity of the rescued human ovarian follicles, which needs to be further evaluated in the future, although one study showed that rescued oocytes can result in healthy offspring in non-human primates (38). Hence, clinical studies need to verify the ability of S1P to prevent toxicant-induced ovarian damage in patients with cancer.

Antioxidants

Reactive oxygen species (ROS) act as second messengers in cell signaling, whose low levels are essential for various biological processes in cells. However, excessive oxidative stress, resulting from the imbalance between ROS production and degradation, leads to DNA damage. Oxidative stress has significant cytotoxicity on ovarian cells such as oocytes and granulosa cells. Chemotherapy drugs can generate large amounts of ROS, which increases oxidative stress and triggers apoptosis in the ovary. In addition, excessive oxidative stress may increase primordial follicles activation and reduce ovarian reserve. Consequently, antioxidants have the ability to eliminate ROS and prevent oxidative stress-induced ovarian damage.

Melatonin Melatonin (N-acetyl-5-methoxytryptamine) is primarily a ubiquitous hormone that is centrally produced by the pineal gland in vertebrates. This molecule is

lipophilic and mainly acts as an antioxidant and free radical scavenger.

Melatonin acts as a powerful antioxidant to prevent free radical damage induced by oxidative stress. Chang *et al.* showed that cisplatin induced the depletion of the follicle pool through the overactivation of dormant primordial follicles in the mouse ovary, and that melatonin administered in co-treatment with cisplatin significantly prevented the loss of primordial follicles in cisplatin-treated ovary (41). In addition, Jang *et al.* demonstrated that the protective effect of melatonin was mediated by suppressing the activation of the PI3K-AKT-FOXO3a (forkhead protein O3) signaling pathway in cisplatin-treated mouse ovary (42). These studies indicate that endogenous melatonin can prevent chemotherapy-induced primordial follicle loss *in vivo*. Furthermore, melatonin is a natural agent with an anti-cancer action that has also been suggested as an adjuvant in combination with cancer therapy. Therefore, melatonin may be a good choice for ovarian protection in chemotherapy.

However, the detailed molecular mechanism of melatonin action regarding the protective response against chemotherapy associated ovarian damage is unclear and requires further study. To assess the efficacy of melatonin, clinical studies should be conducted to prove its protection against chemotherapy-associated ovarian damage as well as the synergistic antitumor effect.

Resveratrol Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a plant-derived natural compound, has a wide variety of biological properties, including anti-inflammatory, anticancer, and anti-aging effects. These effects are mainly attributed to its antioxidant activity as a free radical scavenger.

We have demonstrated that resveratrol (30 mg/kg/day) showed a protective effect against CTX/busulfan-induced oxidative apoptosis of mouse oocytes (43). In this study, resveratrol treatment dramatically upregulated the expression of Nrf2 and SOD2, and attenuated chemotherapy-induced oxidative stress injury. In addition, Atli showed that resveratrol can prevent cisplatin-induced ovarian damage by maintaining the number of primordial and primary follicles in rat by activating Sirtuin1, a deacetylase that acts as a regulator in response to cellular stress (44). In that study, resveratrol was administered intraperitoneally for 14 successive days at fixed time points before cisplatin treatment, and AMH levels were measured 1 week after cisplatin administration. Similarly, in Ozcan's study, resveratrol injections were given 24 h before the administration of cisplatin, and the follicle counts as well as serum AMH levels demonstrated that resveratrol had an antioxidant effect on ovarian damage related to cisplatin-induced oxidative stress in mice (45). Furthermore, many studies have suggested that resveratrol might also represent an anticancer agent, and the tumor-suppressive effects of resveratrol have been proven in various types of cancers. Therefore, resveratrol could be used as an adjuvant in combination with cancer therapy to protect ovarian function.

However, further research is needed to determine the optimum dose and treatment duration to yield the protective effects of resveratrol. Additionally, the results obtained from animal models may not be translatable to humans, and further research on human ovary is required.

MicroRNAs

miRNA are small ncRNA molecules of about 21–22 nucleotides in length and are mainly considered post-

Figure 3

Stem cell-based therapy for ovarian protection. Fertility is protected with iPSCs, MSCs, and ovarian stem cells. A full color version of this figure available via <https://doi.org/10.1530/EJE-20-1178>.

transcriptional gene regulators. miRNA play a vital role in follicle development such as follicular growth, atresia and steroidogenesis. They are also related to DNA damage repair responses during chemotherapy. For example, Zhao *et al.* showed that microRNA (miR)-770-5p promoted cisplatin chemosensitivity by downregulating the expression of DNA repair factor ERCC2 in the human ovarian cancer (46).

Considering the important role of miRNA in follicle development as well as the regulatory effect during chemotherapy, some researchers have attempted to elucidate the role of miRNA in preserving female fertility during chemotherapy. In 2016, Xiao and colleagues found that miR-10a and miR-146 enveloped in exosomes derived from amniotic fluid stem cells could rescue granulosa cells from apoptosis and preserve ovarian follicles after 24-h CTX intervention in mice (47). Fu *et al.* transfected mesenchymal stem cells (MSCs) with miR-21 lentiviral vector and co-cultured them with granulosa cells in phosphoramidate mustard-treated cell culture medium. Then, they constructed rat models of CTX-induced POF, and miR-21 was given on the day of the last CTX injection. Blood samples and ovaries were collected at 15, 30, 45, and 60 days after treatment. They found that miR-21 inhibited the CTX-induced POF by downregulating the expression of PTEN and PDCD4 in the rats (48). Recently, Alexandri *et al.* used a liposome-based system containing let-7a mimic to inhibit chemotherapy-associated ovarian damage in postnatal day 3 ovaries *in vitro* and found that the let-7a mimic prevented the upregulation of genes involved in cell death and reduced the CTX-induced oocyte apoptosis (49).

Based on the previously mentioned results, miRNA may be an interesting method for developing new protective strategies for the female reproduction system during oncological treatment. However, the path to the targeted organ for miRNA is long, complex, and unpredictable, as they face several barriers. For example, miRNA have to avoid phagocytosis or cleavage by endonucleases, and they have to penetrate the membrane of the target cell to exert their function. In addition, stability, immunogenicity, and off-target toxicity must be considered in the treatment. Therefore, these difficulties and challenges must be overcome to introduce miRNA in the field of oncofertility.

Stem cell therapy

Stem cells have general properties such as self-renewal and differentiation. Stem cell-based therapy for ovarian

protection uses induced pluripotent stem cells (iPSCs), MSCs, and ovarian stem cells (Fig. 3). In recent studies, the use of different types of stem cells for treating POF has been reported.

Induced pluripotent stem cells

The generation of functional oocytes from iPSCs should provide a useful model system for improving our understanding of the basic mechanisms underlying oogenesis. In addition, it has potential applications as an alternative source of oocytes for reproduction.

The germ-cell lineage arises from primordial germ cells (PGCs) that undergo a complex multi-step process to form oocytes or sperms. Gametogenesis from iPSCs or embryonic stem cells (ESCs) *in vitro* has been studied for more than a decade. Hubner's group was the first to report the derivation of oocyte-like cells and follicle-like structures from mouse ESCs *in vitro* (50). In 2012, Hayashi *et al.* successfully developed PGC-like cells (PGC-LCs) from mouse ESCs and iPSCs (51). When the PGC-LCs were aggregated with fetal gonadal somatic cells and then grafted to the ovaries of adult female recipients, the cells differentiated into immature oocytes that yielded viable offspring after *in vitro* maturation (IVM) and IVF-ET (51). These methods were further developed for co-culture with fetal gonadal somatic cells *in vitro* for gametogenesis. Furthermore, PSCs were re-derived from the eggs that had been generated, thereby reconstituting the full female germline cycle in a dish. This culture system provides a platform for elucidating the molecular mechanisms underlying totipotency and the production of oocytes of other mammalian species in culture. Parallel studies of human iPSCs are also capable of generating PGC-LCs, and ovarian follicle-like cells can be formed from human ESCs *in vitro* (52). It is possible that technologies involving iPSCs will offer women the opportunity to produce unlimited eggs for assisted reproduction.

However, some limitations also need to be addressed before iPSCs can be used in the clinic to solve human infertility in the future. The first question is the use of human fetal gonadal somatic cells for co-culture *in vitro*. For ethical and legal reasons, it is not realistic at the moment. Other issues relate to nuclear reprogramming and maternal mitochondrial inheritance. Therefore, before iPSCs can be used clinically, we need to eliminate the co-culture of gonadal somatic cells *in vitro* and ensure the normal development of embryos and individuals due to the influence of gene manipulation. Recently, good news from Tian's team showed that germline-competent

iPSCs can be derived from adult mouse granulosa cells by chemical treatment alone, and this chemical reprogramming method could generate functional oocytes and fertile mice and avoid any gene manipulation (53). Therefore, in-depth mechanistic studies in the future may lead to easier obtainment of eggs *in vitro*.

Female germline stem cells

Traditionally, the number of eggs in mammalian ovaries is fixed after birth and is not renewed, only depleted. In 2004, this constraint was lifted by a report that identified the existence of mitotically active germ cells in postnatal mouse ovaries capable of supporting oogenesis after birth (54). In 2009, Zou's team was the first to successfully isolate female germline stem cells (FGSCs) in the ovaries of postnatal mice (55). Subsequently, White's group successfully isolated human oogonial stem cells (OSCs, also termed FGSCs) from reproductive-age ovarian cortex (56). So far, a wealth of evidence on this topic reported from many laboratories support the occurrence of postnatal oogenesis in mammals and elaborate on the features and properties of the OSCs responsible for oocyte formation. It is possible that OSCs will offer women the opportunity to preserve the egg regeneration ability for fertility preservation.

However, the biggest question regarding OSCs is whether the cells really exist, as many researchers have failed to isolate the cells or differentiate them into functional oocytes according to their depicted methods. To strongly confirm the existence of postnatal oogenesis in mammals, the specific biomarkers and culture systems of OSCs should be described in detail and in in-depth research.

Mesenchymal stem cells

MSCs are a kind of PSCs derived from the mesoderm of early embryonic development. MSCs can be isolated from bone marrow, umbilical cord, cord blood, placenta, amniotic membrane, chorionic, and fat tissue; if the differentiation condition is appropriate, they can differentiate into different types of cells.

Some studies have indicated that MSCs can protect the ovary from chemotherapy-induced damage. Wang *et al.* found that injecting human umbilical cord MSCs (hUCMSCs) into mice through the tail vein following CTX treatment could reduce granulosa cell apoptosis, restore ovarian function, and increase sex hormone levels (57). In addition, Song *et al.* found that

hUCMSC therapy 2 weeks after CTX administration could partly protect against CTX-induced POF in a rat model, reducing ovarian cell apoptosis as well as increasing hormone levels and folliculogenesis (58). Furthermore, in the study by Mohamed *et al.*, mice underwent bilateral intra-ovarian administration of human bone marrow MSCs (BMMSCs) suspension 7 days after CTX injection. They found that transplantation of the human BMMSCs increased ovarian weight and reshaped endocrine hormone levels to restore follicular development (59). Adipose-derived stem cells (ADSCs) can also improve the damaged ovarian function induced by CTX in mice. Sun *et al.* injected ADSCs intravenously into a CTX-induced mouse model via the tail vein and evaluated ovarian function 1 week after transplantation. They found that the population of follicles at different stages and ovulation were increased after therapy (60). In addition, Wang's group (61) reported that endometrial MSCs from menstrual blood could both improve ovarian function and restore fertility in a mouse model of chemotherapy-induced POF.

The function of MSCs mainly depends on cell homing and paracrine function, and the therapeutic mechanisms involved in MSCs include migration, anti-apoptosis, anti-fibrosis, pro-angiogenesis, anti-inflammatory, immune regulation, and oxidative stress. In animal studies, there are numerous reports that MSCs can protect against chemotherapy-associated ovarian damage; however, clinical trials of MSCs remain rare. The main reason is that there is a lack of reliable and powerful evidence. In summary, better experimental designs such as large sample, long-term follow-up, and evaluation of the anticancer effect should be implemented to assess the role of MSCs in chemotherapy-associated ovarian damage.

In vitro maturation of primordial follicles

At birth, the human ovary contains about 2 million primordial follicles. At any particular chronological age, the vast majority of oocytes in the ovary are present in these non-growing primordial follicles. Considering the potential risk and low survival rate of follicles after ovary auto-transplantation, IVM of primordial follicles from the earliest stage to maturation and fertilization *in vitro* would be a revolutionary practice for fertility preservation.

Currently, immature mouse oocytes can be activated and matured *in vitro* and *in vivo* (62). However, there remain many technical requirements of IVM in primates and humans, from the primordial follicle to mature,

Figure 4

A summary of the protective methods against chemotherapy-associated ovarian damage. The approach discussed in this article is divided into three parts: the orange background box shows the methods that have been applied in clinical practice; the green background box shows the future potential strategies for protecting against chemotherapy-associated ovarian damage; the blue background box shows the regenerative strategies for chemotherapy-associated ovarian damage. A full color version of this figure is available at <https://doi.org/10.1530/EJE-20-1178>.

fertilizable follicle stage. Several groups have attempted to support early human follicle development *in vitro*, starting with primordial follicle activation. In 2013, Kuwamura *et al.* successfully activated the immature oocytes of POI patients and used them to produce a live birth (63). In that study, they disrupted Hippo signaling by fragmenting ovaries followed by AKT stimulator treatment and autotransplantation. After this discovery, several groups also reported live births from POI patients through this method. In addition, human primordial follicles can be activated and grown to secondary follicles within the loosened ovarian cortical pieces (63). It involves a combination of signal pathways and factors such as the PI3K signal pathway, the Hippo signal pathway, the transcription FOXO3, and mammalian target of rapamycin complex (mTORC).

Therefore, IVM of primordial follicles would be a promising method for women undergoing fertility preservation before being exposed to chemotherapy. However, studies on how the maturation rates of oocytes can be improved and how the normality of these oocytes can be ensured are required.

Artificial ovary

In recent decades, tissue and organ engineering have presented promising means of solving some tough

medical problems that traditional methods cannot, and artificial ovary is one such effort in fertility preservation.

At present, there are no unified definition and application standard for artificial ovary. Some investigators refer to it as 'a natural ovary simulant', in which isolated follicles, ovarian stromal cells, and different growth factors are packaged together inside a biomaterial-based scaffold (64). Another group regards artificial ovary in a biomaterial as a method that can not only preserve fertility but also support normal hormonal function with a significantly decreased risk of introducing malignant cells (65). Ideally and anatomically, the artificial ovary must contain follicles isolated from cryopreserved ovarian tissues and other ovarian stromal cells to provide growth factors (66). It also requires a proper delivery scaffold that is biocompatible, minimally inflammatory, suitable for neo-angiogenesis, and degradable after engraftment to not disturb follicular growth and migration. To date, in animal studies, the artificial ovary has restored endocrine function, achieved *in vivo* follicular development, and resulted in successful pregnancies (67).

The 3D-printed ovaries could allow infertile mice to produce offspring and maintain some ovarian function. However, there is no uniform standard for a 3D reconstruction of an ovary. Different studies have varying follicle types and numbers, bio-scaffolds compositions, and transplantation sites varied among different studies. Other

issues such as immune rejection, surgical procedures, and total cost also need to be considered. Further explorations are needed to improve 3D ovarian scaffolds, optimize the number of cells transferred to the scaffold, and complete the assessment of durable function.

Conclusion

One of the most significant sequelae of exposure to cytotoxic drug chemotherapy is POF and infertility. Hence, protecting the ovarian function has risen to the fore as the primary quality of life issue. The current options for fertility preservation in female patients with cancer include oocyte/embryo/ovarian tissue cryopreservation, and these methods are limited both in their scope (because they are only available to certain populations of patients) and by factors such as delaying the treatment of tumors, increasing the risk of invasion, and metastasis of tumors. Novel strategies that can prevent follicle loss during chemotherapy would yield significant advantages over the existing fertility preservation techniques, as they could be suitable for patients of all ages and life stages and can prevent the endocrine-related adverse effects of POF other than infertility. Based on the methods discussed previously, the summary of ovarian protection and treatment methods in the full text is illustrated in Fig. 4. Even though many unknown and unclear matters remain to be addressed in fertility preservation, many potential protectants and methods have been developed in recent years, acting on different pathways, with effective evidence confirmed in animal model and clinical research, which can offer some future strategies for ovarian protection in female cancer survivors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution statement

Shixuan Wang conceived and coordinated the study; Jiaqiang Xiong, Meng Wu and Liru Xue wrote the manuscript; Ya Li, Jinjin Zhang, Jun Dai, Su Zhou

and Zhiyong Lu revised the manuscript; Weicheng Tang and Dan Chen drew the figure.

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