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Detection of DNA damage in oocytes of small ovarian follicles following phosphoramide mustard exposures of cultured rodent ovaries *in vitro*

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ABSTRACT

Healthy oocytes are critical for producing healthy children, but little is known about whether or not oocytes have the capacity to identify and recover from injury. Using a model ovotoxic alkylating drug, cyclophosphamide (CPA), and its active metabolite, phosphoramide mustard (PM), we previously showed that PM (≥3 μM) caused significant follicle loss in postnatal day 4 (PND4) mouse ovaries *in vitro*. We now investigate whether PM induces DNA damage in oocytes, examining histone H2AX phosphorylation (γH2AX), a marker of DNA double-strand breaks (DSBs). Exposure of cultured PND4 mouse ovaries to 3 and 0.1 μM PM induced significant losses of primordial and small primary follicles, respectively. PM-induced γH2AX was observed predominantly in oocytes, in which foci of γH2AX staining increased in a concentration-dependent manner and peaked 18–24 h after exposure to 3–10 μM PM. Numbers of oocytes with ≥5 γH2AX foci were significantly increased both 1 and 8 days after exposure to ≥1 μM PM compared to controls. Inhibiting the kinases that phosphorylate H2AX significantly increased follicle loss relative to PM alone. In adult mice, CPA also induced follicle loss *in vivo*. PM also significantly decreased primordial follicle numbers (≥30 μM) and increased γH2AX foci (≥3 μM) in cultured PND4 Sprague–Dawley rat ovaries. Results suggest oocytes can detect PM-induced damage at or below concentrations which cause significant follicle loss, and there are quantitative species-specific differences in sensitivity. Surviving oocytes with DNA damage may represent an increased risk for fertility problems or unhealthy offspring.

Introduction

Mammalian females are born with a finite number of gametes, which must remain healthy and viable over a period of decades to maintain their fertility (Hirshfield, 1991; Gougeon, 1998; Faddy, 2000). Primordial germ cells undergo extensive mitoses during fetal organogenesis to establish a limited pool of gametes which arrest in meiosis before becoming incorporated into dormant ovarian primordial follicles (~1 million at birth in humans, (Hirshfield, 1991; Faddy, 2000). The gametes, now considered oocytes, remain in meiotic arrest at the diplotene stage for up to several decades until follicles are either activated to develop and ovulate or undergo atresia, a natural process of elimination (Hirshfield, 1991; Fortune, 2003). From birth, small numbers of follicles are continuously activated to develop, and adult ovaries contain follicles of all developmental stages (Gougeon, 1998). Through this process, a woman will progressively lose follicles throughout her reproductive years until they are depleted, at which time she enters reproductive failure (Hirshfield, 1991; Faddy, 2000).

The integrity of the genome in gametes is critical for the health of offspring. If alterations in DNA of primordial follicles linger in surviving follicles, these changes may remain for decades. Such changes in gamete health could be a mechanism by which exposures lead to detrimental changes in offspring. Evidence for this was observed as an increase in malformations in mice exposed once to CPA (Meirow et al., 2001), although existing clinical and epidemiological studies do not show a higher incidence of birth defects in children of women given chemotherapy and/or radiation (Meirow and Nugent, 2001; Robison et al., 2005; Sklar et al., 2006; Hudson, 2010). This is a major concern for women wishing to have children subsequent to cancer treatments (Byrne et al., 1998; Signorello et al., 2006).

Several factors, including genetics, smoking, and ovotoxic exposures have been determined to accelerate loss of ovarian follicles (Hoyer and Sipes, 1996; Pal and Santoro, 2002). Some of the most severe examples of ovotoxic exposures are anti-cancer therapies. Epidemiological studies have demonstrated that exposure to certain chemotherapy drugs, especially alkylating or DNA-damaging agents, and radiation can result in decreased fertility, temporary or permanent amenorrhea (loss of menstrual cyclicity), and/or

Abbreviations: Ab, antibody; ATM, ataxia-telangiectasia mutated; ATR, ATM-related; CPA, cyclophosphamide; DMSO, dimethyl sulfoxide; DNA-PK, DNA-dependent protein kinases; DSB, double-strand break; IHC, immunohistochemistry; IR, ionizing radiation; PIKK, phosphatidylinositol-3 kinase related kinase family; PM, phosphoramide mustard; PND, postnatal day; γH2AX, phosphorylated histone H2AX.

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reductions in ovarian follicle numbers (Byrne, 1999; Chemaitilly et al., 2006; Hudson, 2010). Advances in treatments against cancer have resulted in increasing long-term patient survival, so avoiding treatment-induced side effects on the reproductive system and fertility has become increasingly important (Linet et al., 1999; Sklar et al., 2006; Brenner et al., 2008). Even if women regain normal ovarian function after chemo- or radiotherapy, they still have a greater risk of undergoing premature reproductive failure than untreated women (Byrne, 1999; Meirow and Nugent, 2001; Robison et al., 2005; Meirow and Schiff, 2005; Sklar et al., 2006; Chemaitilly et al., 2006). Such alterations in fertility suggest toxicity to both growing (acute effects) and primordial (long-term effects) ovarian follicles.

CPA is one of the most commonly used anti-cancer drugs, particularly in children (e.g. leukaemia, Hodgkin's and non-Hodgkin's lymphomas) and women of reproductive age (e.g. breast cancer, bone and soft tissue sarcomas, (Colvin, 1999; Hurley, 2002; Chemaitilly et al., 2006). It is also used as an immunosuppressant for autoimmune diseases, multiple sclerosis and preventing organ transplant rejection (Colvin, 1999). CPA must be activated by cytochrome P-450 enzymes, predominantly Cyp2B1 and 3A4 in the liver, to form the active cytotoxic metabolite, phosphoramide mustard (PM) (Anderson et al., 1995; Ludeman, 1999; Colvin, 1999). PM destroys rapidly dividing cells, such as cancer cells, by binding covalently to DNA, inducing DNA–DNA and DNA–protein crosslinks, and DNA double-strand breaks (DSBs) (Colvin, 1999; Hurley, 2002; Helleday et al., 2008). PM has also been shown to be the active ovotoxic metabolite *in vivo* (Plowchalk and Mattison, 1991) and *in vitro* (Desmeules and Devine, 2006). In cancer patients, CPA can induce rapid amenorrhea, likely from antral follicle destruction or premature ovarian failure, from primordial follicle depletion (Brunner et al., 2006; Chemaitilly et al., 2006; Manger et al., 2006; Sonmezer and Oktay, 2006; Hudson, 2010). In rodents, CPA has species-specific differences in ovarian toxicity, with the most sensitive follicle type being primordial follicles in mice (Plowchalk and Mattison, 1992; Meirow et al., 1999) and antral follicles in rats (Jarrell et al., 1987, 1991; Ataya et al., 1989; Davis and Heindel, 1998).

PM-induced DNA damage may be the mechanism underlying ovarian follicle loss. It may cause DNA DSBs in oocytes, which could lead to permanent changes in gamete health. An early event in the response of cells to DSBs, among the worst types of DNA damage, is phosphorylation of the histone H2AX at Ser139 (Rogakou et al., 1998; Svetlova et al., 2010). H2AX is widely distributed throughout the chromatin, where it is incorporated into the nucleosome around which DNA is wound. Phosphorylation of H2AX (γH2AX) occurs rapidly at sites of DNA DSBs by members of the phosphatidylinositol-3 kinase related kinase family (PIKK), including ataxia-telangiectasia mutated (ATM), ATM-related (ATR) and DNA-dependent protein kinases (DNA-PK), which become activated in response to DSBs (Burma et al., 2001; Durocher and Jackson, 2001; Bakkenist and Kastan, 2003; Svetlova et al., 2010). γH2AX plays an important role in recruiting and maintaining DNA repair molecules at sites of damage until repair is complete, and has become the gold standard for localizing DSBs (Paull et al., 2000; Fernandez-Capetillo et al., 2003; van Attikum and Gasser, 2005; Svetlova et al., 2010). γH2AX has been observed in cell lines after toxic exposures, during apoptosis, during sister-chromatid exchange, and even in human oocytes following exposure to ionizing radiation or high-dose chemotherapy exposure (Paull et al., 2000; Fernandez-Capetillo et al., 2004; Suh et al., 2006; Kato et al., 2008).

We have previously shown that single exposures of cultured mouse ovaries to ≥3 μM PM induced primordial and small primary follicle depletion (>90%), with morphological changes specifically in oocytes of these follicles as early as 24 h following exposures (Desmeules and Devine, 2006). Given the mechanism of action of CPA in treating cancers includes DNA damage, and the response of oocytes to DNA damage has been poorly studied, we examine the possibility that the mechanism by which PM induces primordial and primary follicle loss involves DNA DSBs in oocytes. Furthermore, we test whether or not damaged primordial follicles might remain in the ovary.

Materials and methods

Animals. Breeding colonies of CD-1 mice and Sprague–Dawley rats (Charles River, Montréal, QC) were maintained to produce pups for use in *in vitro* ovarian culture experiments. Animals in colonies were replaced every 6 months. Ovaries were collected from PND4 female pups (day of birth designated as day 0). For *in vivo* experiments, 35-day-old adult female CD-1 mice were used. Animals were allowed to acclimate for 5 days before experiments began. Rodents were housed in plastic cages under a 12 h light/dark photoperiod and received food and water *ad libitum*. All experimental procedures were approved by the INRS Animal Care and Use Committee and conformed to Canadian Council on Animal Protection guidelines.

In vitro ovarian culture and ovotoxic exposures. Whole PND4 ovaries were cultured *in vitro* as previously described (Devine et al., 2004; Desmeules and Devine, 2006). Culture medium was replaced every 2 days. Exposures were initiated on day 4 of culture, and chemicals were removed with the subsequent replacement of culture medium. Ovaries were then cultured for up to 8 days following start of exposures. PM (cyclohexylammonium salt; National Cancer Institute, Bethesda, MD), was dissolved in culture medium immediately before exposures. Wortmannin (Wtn; Sigma Chemical Co., Oakville, ON) was dissolved in dimethyl sulfoxide (-DMSO), diluted in culture medium, and applied 8 h after the start of PM exposure. The timing chosen for these exposures was due to the short half-life reported for Wtn in culture (Sarkaria et al., 1998). DMSO was also added to wells of control ovaries to concentrations (0.5%) equal to that in wells with Wtn in experiments with Wtn. For exposures to radiation, ovaries were placed in Petri dishes and exposed to a ^{137}Cs source at a dose rate of 6.3×10^{-2} Gy/s (Gamma Cell; Atomic Energy Canada, Ottawa, ON), then put back into culture plates and fixed 2 days later. Three to six experiments of each type were performed.

Ovaries were fixed at specific time points after exposures in either Bouin's fixative (fixation for 2–4 h) for follicle counts or in 3.7% buffered formaldehyde for fluorescent immunohistochemistry (IHC, fixation for 4–24 h).

In vivo exposures. A single intraperitoneal injection of 0.9% saline (vehicle) or cyclophosphamide (75, 150 or 250 mg/kg in 0.9% saline; Sigma) was given to 40-day-old mice. Doses were chosen based upon the literature (Plowchalk and Mattison, 1992; Meirow and Nugent, 2001). Groups of mice were then sacrificed 1 or 8 days after exposure (3–5 per treatment group per time point). At necropsy, body and organ weights were recorded. One ovary from each mouse was preserved in Bouin's fixative for follicle counts and the other in buffered 3.7% formaldehyde for IHC. Experiments were performed 3 times.

Ovarian follicle counts. Bouin's-fixed ovaries were embedded in paraffin following standard histological procedures. Tissue sections (5 μm) were cut and stained with haematoxylin and eosin, and ovarian follicle populations were determined as described previously for cultured (Desmeules and Devine, 2006) or adult ovaries (Mayer et al., 2004) in every 12 or 20th section, respectively. Healthy follicles were counted separately from atretic follicles. Atretic follicles were identified as those that had pyknotic (eosinophilic) oocytes or ≥3