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Cellular and molecular mechanisms of chemical-induced ovarian toxicity

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Cellular and molecular mechanisms of chemical-induced ovarian toxicity

by

Shanthi Ganesan

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Toxicology

Program of Study Committee:
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Iowa State University
Ames, Iowa
2014

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DEDICATION

To all the animals who we have sacrificed their innocent lives for the betterment of human beings.

Additionally, I dedicate this thesis to my family and kids who have supported me in my research endeavors.
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ABSTRACT

The ovary is the ovum-producing female reproductive organ composed of follicles at different stages of development. Chemicals which selectively damage large growing or antral follicles only temporarily interrupt reproductive function because these follicles can be replaced by recruitment from the greater pool of primordial follicles. However, chemicals that destroy oocytes contained in primordial follicles can cause permanent infertility and premature ovarian failure (early menopause in women), since once a primordial follicle is destroyed, it cannot be replaced. We hypothesized that ovotoxic chemicals can bind to DNA to induce DNA damage and that the ovarian cells activate DNA repair, cell cycle arrest and apoptosis to cause chemical-induced toxicity. Additionally, we investigated cell to cell communication as targets of ovotoxicants. Gap junction proteins are involved in folliculogenesis and we questioned whether an external factor such as ovotoxicant exposure and obesity alters gap junction proteins to cause follicle loss. To test this hypothesis, ovarian mRNA and protein expression profiles involved in DNA repair, cell cycle arrest, apoptosis and gap junction formation were quantified using qRT-PCR, western blotting or immunohistochemistry techniques after exposure to two different chemicals: Phosphoramide mustard (PM) and 7,12-dimethylbenz[a]anthracene (DMBA). In addition, we investigated the impact of PM and/or DMBA in three physiological paradigms: 1) spontaneously immortalized rat granulosa cells (SIGC); 2) In vitro neonatal ovarian culture system; 3) In vivo obese mouse model. Overall, our data demonstrates that the ovarian DNA damage responses are activated and up-regulated in both a dose- and time-dependent manner. Also, chemical exposure alters the gap junction protein expression in ovaries, interfering with inter-cellular communication, leading to follicular demise. Taken together, our discoveries
detailed herein shed new mechanistic light on the events that precipitate ovarian follicle depletion leading to female infertility, and potential targets for prevention of chemical-induced infertility are described.
ORGANIZATION OF THE DISSERTATION

This dissertation is organized into 9 chapters and an appendix. Chapter 1 is the general introduction to the research. In Chapter 2, we hypothesized that DMBA induces DNA double strand breaks and that the ovary activates repair mechanisms to protect the gamete against DMBA-induced DNA damage, leading to follicle loss. Our results demonstrated that DMBA induces ovarian DNA damage and those DNA repair mechanisms are induced as a potential mechanism to prevent follicle depletion, thus confirming our hypothesis. Chapter 3 focuses on the impact of obesity on the ovarian response to DMBA exposure in mice. Since obesity is associated with increased incidence of offspring birth defects, we hypothesized that a DMBA-induced DNA damage repair response might be compromised in ovarian tissue from obese females. This study showed that DMBA exposure induced DNA damage as well as the ovarian DNA repair response, further supporting that DMBA causes ovarian DNA damage as an ovotoxic insult in an in vivo model. Additionally, the ovarian DNA repair response was partially attenuated in obese females raising concern that obesity may be an additive factor during chemical-induced ovotoxicity. Chapter 4 describes the impact of chemical exposure on the gap junction (GJ) connexin (CX) protein in neonatal cultured rat ovaries. We hypothesized that CX protein level might be altered by DMBA exposure during follicle loss, contributing to loss of follicular viability. We found that DMBA exposure impacts ovarian CX protein prior to and at the time of follicle loss. It is possible that such interference in follicular cell communication is detrimental to follicle viability, and may play a role in DMBA-induced follicular atresia. Chapter 5 details the impact of obesity on ovarian CX GJ proteins during DMBA exposure in a murine in vivo model. Since DMBA and obesity altered GJ protein expression in extra ovarian tissues, this study investigated both the impact of
obesity and DMBA exposure on ovarian CX protein levels using the lethal yellow mouse model of progressive obesity. This study showed that that obesity temporally alters GJ protein abundance and that DMBA-induced ovotoxicity may involve reduced GJ protein function. Chapter 6 describes our work to identify PM-induced DNA adduct formation and activation of the DNA damage repair response in rat ovarian granulosa cells. PM was found to form DNA adduct in ovarian granulosa cells to induce DNA damage and elicit the ovarian DNA repair response. Chapter 7 investigated on signaling mechanisms of PM-induced ovarian follicle loss, and demonstrated that PM-induced follicle loss may occur through an E2F1-P73 dependent mitochondrial apoptotic pathway. Additionally results from protein kinase C (PKCδ) deficient mice indicated that PKCδ is essential for survival of ovarian follicles, but that no additional impact of PM was found in these mice. ATM can act as a pro-apoptotic factor at the time of PM-induced follicle loss and ATM inhibition improve the survival of ovarian follicles. In Chapter 8, we hypothesized that obesity might alter PM-induced DNA repair mechanism in ovaries. Our data indicate that PM induces ovarian DNA damage in in vivo model as a mode of ovotoxicity and that the ovary is responsive to such an insult and activates the DNA repair response. Additionally, and of concern, obesity altered the ovarian response to an ovotoxic exposure. Chapter 9 presents the general conclusion for all these studies. In the appendix, some results which were not included in the manuscripts prepared for publication to date are summarized.
CHAPTER 1. GENERAL INTRODUCTION

The Ovary

The female reproductive system is comprised of ovaries, fallopian tubes, uterus, vagina, vulva, mammary glands and breasts. These organs are essential for the production and transportation of gametes and the production of sex hormones. Also, the female reproductive system facilitates the fertilization of ova by sperm and supports the development of offspring during pregnancy.

The ovaries are a pair of small glands located on the left and right sides of the pelvic body cavity lateral to the superior portion of the uterus. The ovary produces female sex hormones such as estrogen and progesterone as well as ova (commonly called "eggs"), the female gamete. The ovary contains two important endocrine glands: the follicle and the corpus luteum. The dominant follicle(s) is essential for the production of estrogens which controls the development of the mammary glands and uterus during puberty and stimulates the development of the uterine lining during the menstrual cycle. The corpus luteum is derived from follicular tissue following ovulation and required for progesterone production which acts on the uterus during pregnancy to allow the embryo to implant and develop in the womb.

Follicular Development

Folliculogenesis describes the progression of primordial follicles into large pre-ovulatory follicles, in which the oocyte is maintained in meiosis I of oogenesis. The follicles containing germ cells, or oocytes, develop from primordial germ cell (PGC) populations which are established during the embryonic period and are motile and invasive (Witschi,
1948). The somatic components of the follicles (granulosa, theca, endothelial cells and connective tissue) are derived from the embryonic indifferent gonad (Yoshinaga et al., 1988; Hirshfield, 1991). After germ cells invade the indifferent gonad, both cell types undergo extensive hyperplasia. The germ cells lose their motile characteristics and proliferate rapidly for 15-16 h in mouse to form 85,000 germ cells (Tam and Snow, 1981; Wylie et al., 1986). There are approximately 75,000 germ cells in rat ovary (Beaumont and Mandl, 1963). While the germ cells are undergoing mitosis, the somatic cells of the gonad proliferate rapidly also (Hirshfield, 1991).

The somatic cells continue to proliferate throughout the embryonic period, gradually enveloping individual oocytes and forming primordial follicles. Each primordial follicle consists of a single oocyte surrounded by a few flattened somatic cells, referred to as “pre-granulosa cells” (Gondos, 1970). The time of appearance of the first primordial follicles in rats are the first day postpartum and in mice the second day postpartum (Ueno et al., 1989). After formation of primordial follicles, pre-granulosa cells cease to divide and enter a period of quiescence and the oocytes remain arrested in the first meiotic prophase. In rats, the primordial follicle contains approximately 4 granulosa cells in its gross section (Hirshfield and Schmidt, 1987). The first sign of growth is the resumption of cell proliferation by squamous granulosa cells and later stages of growth can be recognized by an increase in the size of the oocyte and a change in shape and number of the granulosa cells (Lintern-Moore and Moore, 1979). Primordial follicles provide the pool for recruitment of developing follicles; therefore, they are a fundamental reproductive unit within the ovary. Some primordial follicles leave the quiescent state relatively quickly after they are formed, and some are dormant for months or years. The process by which primordial follicle activation
occurs is called “initial recruitment” which is mediated by the counterbalance of various stimulatory and inhibitory hormones and locally produced growth factors (Fortune et al., 2000).

The first sign of oocyte growth in primordial follicles is alteration of the surrounding squamous (flattened) granulosa cells into cuboidal-shaped cells which are around 8 granulosa cells in its gross section in the primary follicular stage (Hirshfield, 1991). The oocyte genome is activated and genes become transcribed. Rudimentary paracrine signaling pathways that are vital for communication between the granulosa cell and oocyte are formed (Gilchrist et al., 2008). Both the oocyte and the follicle grow dramatically, increasing to almost 100 µm in diameter. Once the follicle makes this transition from primordial to primary, other structural changes occur such as development of the zona pellucida and acquisition of the theca layer (Hirshfield, 1991). The zona pellucida is a glycoprotein polymer capsule which forms around the oocyte, separating it from the surrounding granulosa cells to provide protection for the oocyte as well as to provide attachments for the specialized inner layer of granulosa cells, known as “cumulus cells” (Richards, 1980; Hirshfield, 1991). At this stage, another layer of specialized somatic cells begin to proliferate and form a shell outside the basement membrane enclosing the oocyte and granulosa cell layer. These cells appear in concentric rings surrounding the follicle and are designated theca interna cells (Peters, 1969). Theca cells provide two important functions: (1) attachment of arterioles for the development of an independent blood supply and (2) secretion of progestins and androgens to regulate follicle development (Hirshfield, 1991). The zona pellucida, which remains with the oocyte after ovulation, contains enzymes that catalyze with sperm to allow penetration. Primary follicles are remaining gonadotropin-independent until the antral stage.
The oocyte contained in a secondary follicle has a large, spherical nucleus (germinal vesicle) that grows in proportion to the growth of the oocyte and the follicular cells continue to proliferate and form a second layer around the oocyte. The layers of granulosa cells surrounding the oocyte increase rapidly to reach follicular diameters of up to 250 μM. Gap junctions (GJ) are formed between individual cells in the granulosa cell layer to facilitate intercellular transport of nutrients and metabolites to the oocyte (Goodenough et al., 1996). A rise in pituitary FSH caused by the disintegration of the corpus luteum from the previous follicular cycle, precipitates the selection of follicles. The selected follicles, called antral follicles, compete for growth-inducing FSH. At that point an antrum begins to form within the granulosa cell layer and this fluid-filled cavity enlarges as the final stages of follicular development are reached (Shalgi et al., 1973). The number of follicles that reach the antral stage is quite small. In response to the rise of circulating FSH, the antral follicles begin to secrete estrogen and inhibin, which have a negative feedback effect on FSH (De Ziegler et al., 2007). Follicles that have fewer FSH-receptors will not be able to develop further; they will show retardation of their growth rate and become atretic. Collectively, primordial, primary and secondary follicles are less than 250 μm in diameter and are referred to as pre-antral follicles. Eventually, only one follicle will be viable and this remaining follicle, called the dominant follicle (mono bearing species), will grow quickly and dramatically up to 20 mm in diameter to become the pre-ovulatory follicle. In the pre-ovulatory follicle, the basic structure of the mature follicle remains unchanged and no novel cells are detectable. Granulosa and theca cells continue to undergo mitosis concomitant with an increase in antrum volume. Pre-ovulatory follicles can attain a tremendous size that is hampered only by the availability of FSH, thus, it is now dependent on gonadotrophins (Farmer et al., 2005).
Under action of an oocyte-secreted morphogenic gradient, the granulosa cells of the tertiary follicle undergo differentiation into four distinct subtypes: corona radiata, surrounding the zona pellucida; membrana, interior to the basal lamina; peri-antral, adjacent to the antrum and cumulus oophorous, which connects the membrana and corona radiata granulosa cells together. Theca cells express receptors for luteinizing hormone (LH) which induces the production of androgens by the theca cells, most notably androstenedione, which are aromatized within granulosa cells to produce estrogens, primarily estradiol (Richards et al., 1976; Richards, 1980). In humans, 1-2 pre-ovulatory follicles develop approximately every 28 days, whereas, in rats, 6-12 follicles develop every 4-5 days (Richards, 1980). By the end of antral stage, however 99.9% of antral follicles will have undergone atresia (Barnett et al., 2006) (Figure 1).

By the end of the follicular, or proliferative, phase of the menstrual cycle, the cumulus oophorus layer of the pre-ovulatory follicle will develop an opening, or stigma, and expulse the oocyte with a complement of cumulus cells in a process called ovulation (Niswender et al., 1972). The oocyte is now called the ovum and is competent to undergo fertilization. The ovum will now travel down one of the fallopian tubes to eventually be discharged through menstruation, if not fertilized by a sperm cell, or implanted in the uterus, if fertilized. The fully developed oocyte (gamete) is now at the behest of the menstrual cycle. The corpus luteum develops from an ovarian follicle during the luteal phase of the menstrual cycle or estrous cycle, following the release of a secondary oocyte from the follicle during ovulation (Mackenzie, 1922; Parkes, 1928). While the oocyte (later the zygote if fertilization occurs) traverses the fallopian tube into the uterus, the corpus luteum remains in the ovary. The corpus luteum is typically very large relative to the size of the ovary; in humans, the size of
the structure ranges from under 2 cm to 5 cm in diameter. The development of the corpus luteum is accompanied by an increase in the level of the steroidogenic enzyme cytochrome P450scc that converts cholesterol to pregnenolone in the mitochondria (Rapoport et al., 1998). Pregnenolone is then converted to progesterone that is secreted out of the cell and into the blood stream and it is essential for maintenance of pregnancy.

Throughout the reproductive life span of a female, the total number of primordial follicles selected to develop for ovulation is small compared to the total population. Instead, the vast majority are lost to attrition in various stages of development by a process called atresia. This process of follicle death is characterized by radical apoptosis of all constituent cells and the oocyte. Although it is not known what causes atresia, the presence of high concentrations of FSH has been shown to prevent it (Peluso and Steger, 1978). The ovarian content of oocytes is dynamic and fluctuates with age and it reaches peaks during embryonic development. In humans, the peak number of oocytes ever present, about seven million, occurs at five months gestation, at birth the number has dropped to two million, 250,000-400,000 at puberty, and no functional oocytes remain at menopause (Hirshfield, 1991). During the lifetime of a woman, ovulation only accounts for 400–600 oocytes. Therefore, of the primordial follicles a female is born with, greater than 99% will be lost by atresia at various stages of development.

**Gap Junction Proteins**

A gap junction (GJ) or nexus is a specialized membrane domains (White and Paul, 1999), composed of collections of channels that directly connect the cytoplasm of two cells and allows various molecules, amino acids, nucleotides, and second messengers and ions to
pass freely between cells (Pitts and Simms, 1977; Lampe and Lau, 2000; Lampe and Lau, 2004).

GJ channels are oligomeric structures made up of connexin (CX) subunits (Beyer and Berthoud, 2009). CX’s are four-pass transmembrane proteins with both C and N cytoplasmic termini, a cytoplasmic loop (CL) and two extra-cellular loops, (EL-1) and (EL-2) (Tripathi and Tripathi, 2010) (Figure 2). CX’s are assembled in groups of six to form hemichannels, or connexons, and two hemichannels then combine to form a GJ (Beyer et al., 2013) (Figure 3). The Cx gene family is diverse, with twenty-one identified members in the sequenced human genome, and twenty in the mouse (nineteen of which are orthologous pairs). They usually weigh between 26 and 60 kDa, and have an average length of 380 amino acids. The various CXs have been observed to combine into both homomeric and heteromeric GJ, each of which may exhibit different functional properties including pore conductance, size selectivity, charge selectivity, voltage gating, and chemical gating (Saez, 2003; Harris, 2007).

Several GJ channels (hundreds) assemble within a macromolecular complex called a GJ plaque (Spray and Scemes, 2013). GJs are involved in the regulation of cellular growth, metabolism, differentiation (Sohl and Willecke, 2003; Wei et al., 2004) and ovarian folliculogenesis (Simon et al., 1997). Granulosa:granulosa and granulosa:oocyte cell to cell communication are necessary for maintenance of follicular viability. Oocyte growth and development depends on upon the supply of nutrients transmitted from follicle cells via GJ’s. Interconnection by GJ’s was observed between the innermost layer of cumulus and the oocyte, between the adjacent cumulus cells, between the granulosa cells and also between cumulus and granulosa cells (Dekel, 1987). In the mouse genome, nearly 20 Cx genes have been identified and at least five members of the CX family are localized to the rodent ovarian
follicle: CX26, CX32, CX37, CX43, and CX45 (Kidder and Mhawi, 2002). The most abundant gap junction proteins expressed in the mice ovaries are CX37, CX43 and CX45 (Simon et al., 1997; Ackert et al., 2001; Wright et al., 2001).

The most abundant GJ protein in granulosa cells is CX43 and its expression is elevated as follicle size increases in response to FSH (Beyer et al., 1989; Valdimarsson et al., 1993; Okuma et al., 1996). In addition to granulosa cells, CX43 has been noted in rat oocytes (Granot et al., 2002). C57BL/6 mice lacking CX43 exhibit follicular developmental arrest at the primary follicle stage, indicating a failure of granulosa cells to proliferate (Ackert et al., 2001). WNT signaling is necessary during maturation of the ovarian follicles and Wnt4 knockout mice had found reduced (30%) expression of CX43 compared to that of controls (Prunskaite-Hyyryläinen et al., 2014). The WNT2/CTNNB1 pathway regulates CX43 expression and GJ intracellular communication in granulosa cells by modulating CTNNB1 stability and localization in adherens junctions. Furthermore, Ctnnb1 knockdown interfered with the ability of FSH to promote mobilization of CX43 into GJ’s (Wang et al., 2013). FSH induces the development of the clock system by increasing the abundance of CX43 in rat granulosa cells (Chen et al., 2013). High levels of androgen reduce CX43 abundance and impair GJ intercellular communication between human granulosa cells through androgen receptors (Wu et al., 2010a). Cx43 and Cx45 transcripts were judged too static to be a regulator of GJC, while CX43 protein expression was highly responsive to gonadotropins, suggesting that it might be the principal regulator of GJ communication (GJC) (Santiquet et al., 2013). CX43 was decreased in association with atresia supporting the concept that loss of GJC plays a coordinating role in the process of atresia (Feranil et al., 2005). CX43 levels are increased in granulosa cells following activation of follicular growth and maturation (Melton
et al., 2001), while reduced granulosa cell expression of CX43 is linked to elevated apoptosis in porcine, bovine (Johnson et al., 1999; Cheng et al., 2005) and avian (Krysko et al., 2004) species. CX45 has also been identified as a component of GJs between granulosa cells, where it co-localizes with CX43 in some plaques (Okuma et al., 1996; Kidder and Mhawi, 2002).

Heterocellular GJs between oocytes and granulosa cells contain CX37, and there is evidence that CX37 is expressed both by the oocyte and by the adjacent granulosa cells (Simon et al., 1997). CX37 is thought to be involved in follicular development and ovulation as well as luteal tissue growth, differentiation, and regression (Borowczyk et al., 2006). Ablation of Cx37 in mice results in the loss of oocyte-granulosa cell dye-transfer and the failure of follicle to progress to the antral stage. In addition, oocytes in the follicles of these mice are small and fail to reach meiotic competence, and follicles as a whole undergo premature luteinization (Simon et al., 1997; Carabatsos et al., 2000). Both follicle development and oocyte maturation were impaired in ovaries containing either wild-type oocytes and Cx43-deficient granulosa cells or Cx37-deficient oocytes and wild-type granulosa cells (Gittens and Kidder, 2005). After cryopreservation and subcutaneous transplantation of ovarian tissue, proteins forming GJ between oocytes and granulosa cells are under-expressed compared with normal controls (Lee et al., 2008). Also, continuous activation of FOXO3a in oocytes caused a dramatic reduction in the expression of bone morphogenic protein 15 (BMP15), CX37 and CX43, all of which contribute to the establishment of paracrine and GJC in follicles (Liu et al., 2007). These studies indicate that at least three CXs: CX43, CX45 and CX37, are necessary for normal folliculogenesis.

CXs are also targets for a number of chemicals including retinoids, carotinoids chemotherapy (King and Bertram, 2005), cigarette smoke components (McKarns et al.,
2000) and poly aromatic hydrocarbons (PAH) like benzo/a/pyrene (BaP), benzo/e/pyrene (BeP), benz/a/anthracene (BA), dibenz/ah/anthracene (DBahA), 3-methylcholanthrene (3-MC), phenanthrene (PA) (Sharovskaya et al., 2006). In utero flutamide exposure reduces Cx43 mRNA and protein expression during the rat embryonic period (Cai et al., 2014). Oocyte-directed depletion of Cx43 using the Cre-LoxP system leads to subfertility in female mice (Gershon et al., 2008). Lindane suppresses FSH and transforming growth factor beta-induced CX43 GJ formation and steroidogenesis in rat granulosa cells (Ke et al., 2005). CXs are also altered in mesenteric arteries of insulin resistant obese rat (Young et al., 2008). A high fat diet reduces the CX expression in the heart tissue of female rats which increase the risk of ventricular arrhythmia (Aubin et al., 2010). These studies indicate that GJ proteins are not only altered by chemicals but also potentially by obesity.

**Obesity**

Obesity is a health issue of epidemic proportions worldwide and in the United States. In 2008, it was estimated that up to 10% of all medical spending in the United States was attributed to the medical burden of obesity (Finkelstein et al., 2009). Obesity is a particularly important concern in the health of women because 64.1% of women in the United States (Flegal Km et al., 2010) and 30% of reproductive age women are obese or overweight (Boney et al., 2005). Obesity is associated with menstrual cycle disturbances, ovulatory dysfunction, infertility, decreased conception, early pregnancy loss and congenital abnormalities (Cardozo et al., 2012; Sauber-Schatz et al., 2012). These reproductive sequelae result from the effects of obesity on a number of different steps in the reproductive process, including ovarian follicular recruitment, oocyte development and quality, oocyte fertilization,
and embryo development and implantation (Jungheim et al., 2013). Epidemiologic investigation of obesity and time to pregnancy demonstrated that the time to spontaneous pregnancy was increased among obese women including those experiencing regular ovulation (Gesink Law et al., 2007). Obese women (OW) undergoing treatment with assisted reproductive technology (ART) require significantly higher doses of gonadotropin than normal-weight women (NWW) to produce a similar number of ovarian follicles during controlled ovarian hyperstimulation (Jungheim and Moley, 2010).

Mature oocytes from OW are less likely to be fertilized than oocytes from NWW, suggesting that oocytes from OW are of poorer quality (Shah et al., 2011). Compared with NWW, OW are less likely to achieve a clinical pregnancy after in vitro fertilization (IVF) (Jungheim et al., 2009), are at higher risk of miscarriage after an ART conception (Rittenberg et al., 2011a; Rittenberg et al., 2011b) and are less likely to achieve a live birth after IVF (Luke et al., 2011). These findings may be attributable to poor embryo quality among OW (Metwally et al., 2007). Lipotoxicity is a mechanism by which obesity may impact oocyte quality (Wu et al., 2011) and induces DNA damage in obese men (Dupont et al., 2013b) further contributing to infertility. Women with normal weight and body mass index, but high fat mass (> 30%) designated as normal-weight obese syndrome (NWO) (Di Renzo et al., 2006), in which the concentration of plasma pro-inflammatory cytokines and oxidative stress markers are higher than in non-obese women (Di Renzo et al., 2010). Alkali labile sites were higher in obese women compared to NWO and 8-oxo-dG level was also slightly higher in NWO (Tomasello et al., 2011). Maternal obesity also has adverse effects as early as the oocyte and pre-implantation embryo stage which may contribute to lasting morbidity in their offspring (Jungheim et al., 2010). Understanding obesity and its impacts
on female reproductive function is important because future generations, namely, the children of obese women, ultimately will be affected.

High body mass index has also been associated with increased risk for various cancers induced by 7,12-dimethylbenz[a]anthracene (DMBA) exposure, including breast cancer in female zucker rats (Hakkak et al., 2005). Brca1 and Brca2, down-regulated in human breast cancer cells by DNA damaging agent (Andres et al., 1998), are crucial members of the ATM-mediated DSB repair family of genes and mutation in these genes are associated with increased risk of ovarian, breast and other cancers (Robson et al., 1998; Kauff et al., 2002). Women who carry germ line mutations in the Brca1 gene, show low response to ovarian stimulation while undergoing fertility preservation by oocyte or embryo cryopreservation (Oktay et al., 2010). Recent research also suggests that women with Brca1 mutations may experience earlier menopause (Rzepka-Górksa et al., 2006). Impairment of DNA DSB repair was associated with accelerated loss of ovarian follicular reserve and accumulation of DSB in human oocytes which also implicate DNA DSB repair efficiency as an important determinant of oocyte aging in women (Titus et al., 2013).

**Ovarian Toxicity**

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The stage of development at which the follicle is targeted by an ovotoxicant determines the impact of chemical exposure on reproduction (Hoyer and Sipes, 1996). Damage to large growing or antral follicles can cause a disruption of cyclicity by impacting ovarian steroid production and ovulation. Chemicals which selectively damage large growing
or antral follicles may only temporarily interrupt reproductive function because these follicles can be replaced by recruitment from the greater pool of primordial follicles. If an ovotoxic chemical destroys oocytes of primordial follicles, which can cause permanent infertility and premature ovarian failure will ensure, since once a primordial follicle is destroyed, it cannot be replaced. Destruction of oocytes contained in primordial follicles may have a delayed effect on reproduction until such a time that recruitment for the number of growing and antral follicles can no longer be supported (Generoso et al., 1971; Hooser, 1994). Ultimately, if the ovary is depleted of primordial follicles, ovarian failure occurs, defines as cessation of ovarian cyclicity. The average age of menopause in the United States is 51, and is a direct consequence of depletion of the follicular reserve. Menopause has been associated with a variety of health problems in women. These include increased incidences of osteoporosis, cardiovascular disease, depression and Alzheimer’s disease (Avioli, 1981; Paganini-Hill and Henderson, 1994; Sowers and La Pietra, 1995). Additionally, in laboratory animals, premature ovarian failure is associated with an increased incidence of ovarian neoplasms (Hoyer and Sipes, 1996) (Figure 4). A variety of environmental factors have been highly correlated with early menopause in women (Everson et al., 1986; Cooper et al., 1995). Therefore, as a woman ages, her overall health is affected by the onset of menopause, and this can be further impacted by environmental factors to which she has been exposed (Hoyer and Sipes, 1996). A number of chemical classes can deplete ovarian follicles and altered steroidogenesis, leading to impaired ovarian function and infertility, including but not limited to environmental, industrial, chemotherapeutic and xenoestrogenic chemicals (Hoyer and Sipes, 1996).
Chemotherapy for cancer treatment uses chemical substances in single or combined form to cure the cancer, prolong the life or to reduce the symptoms. For many childhood cancers, the 5-year survival rate is now 70% with survival rates in adult cancers also increasing (Thomson et al., 2002). The most common malignancy in adult women is breast cancer, affecting one in nine women, with an estimated 25% of these women bring pre-menopausal at diagnosis (Stearns et al., 2006). Given this success, clinical concern in good-prognosis malignancies can now also focus on the long-term adverse effects of chemotherapy. The main reproductive side effects associated with chemotherapy in female survivors include early menopause and an increased infertility rate in women who maintain ovarian activity after chemotherapy (Letourneau et al., 2012). The offspring of treated individuals can potentially also be affected by early embryonic mortality through oocyte chromosomal aberration (Gonfloni et al., 2009). Studies have shown that exposure to chemotherapy drugs such as alkylating or DNA damaging agents cause 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). Female infertility by chemotherapy appears to be secondary to premature ovarian failure by loss of primordial follicles but this is not necessarily a direct effect of the chemotherapeutic agents. Instead, the disappearance of primordial follicles could be due to increased rate of growth initiation to replace damaged developing follicles (Keating et al., 2009; Morgan et al., 2012). Likewise, the loss of oocytes need not necessarily be a direct result of damage: evidence suggests that chemotherapy drugs can also induce oocyte death indirectly via damage to somatic cells (Morgan et al., 2012) (Figure 5).
Cyclophosphamide (CPA) is chemotherapeutic drug used to treat cancer and autoimmune diseases in human. 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; Hudson, 2010). CPA significantly reduced the number of primordial and antral follicles in C57BL/6N and DBA/2N mice and in Sprague-Dawley (SD) rats (Shiromizu et al., 1984; Plowchalk and Mattison, 1991). Mice treated with CPA in low doses for 1 year found reduced numbers of oocytes (especially primordial) and corpora lutea with no effect on other tissues such as the kidney, spleen, thymus or lymph nodes (Miller and Cole, 1970). Estrous cyclicity was destroyed and cysts/tumors were observed in the ovarian germinal epithelium. CPA destroyed primordial follicles but left larger follicles intact in Balb/c mice (Meirow et al., 1999). Also time- and dose-dependent relationship between CPA and ovarian toxicity was observed by looking at changes in ovarian structure and function (Plowchalk and Mattison, 1992). In C57BL/6N mice given a single i.p. injection of CPA (75, 200, or 500 mg/kg), primordial follicle numbers were significantly reduced to 73, 42 and 38 percent of controls, respectively. The loss of primordial follicles was essentially complete in three days, and the estimated ED50 (concentration that produced 50 percent follicle loss) was 122 mg/kg body weight (Plowchalk and Mattison, 1992). From these results it appears that premature ovarian failure in women treated with CPA is likely to be via destruction of primordial follicles. Interestingly, in a rat study, it was shown that a single injection of CPA caused damage to growing and antral follicles, but spared an effect on primordial follicles, because cyclicity was disrupted within a week, but had been restored 2 weeks post-exposure (Jarrell et al., 1991). Therefore, the impact of CPA on ovarian follicles may be species dependent.
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 be able to 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 and Nugent, 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; Sklar et al., 2006; Hudson, 2010). This remains, however, is a major concern for women wishing to have children subsequent to cancer treatments (Signorello et al., 2006).

CPA is a precursor for the active form of the drug and must be bioactivated to exert its functional activity (Brock, 1976). The active metabolites include 4-hydroxy-CPA, aldophosphamide and phosphoramidemustard (PM), which is thought to be the active anticancer agent (Boddy and Ratain, 1997; Boddy and Yule, 2000). Although the mechanisms underlying the cytotoxic and teratogenic properties of CPA are not completely understood, it is generally believed that CPA-induced toxicity is related to the induction of DNA-DNA interstrand cross-links by PM (Brookes and Lawley, 1961). The initial reaction leading to the formation of DNA-DNA cross-links is alkylation at the N-7 position of guanine. This initial PM-DNA adduct is relatively unstable and converted to the corresponding nor-nitrogen mustard adducts, one of which is the N, N-bis [2-(N7-guaninyl) ethyl] amine (G-NOR-G) cross links (Hemminki, 1985). Tentative evidence, based upon alkaline elution studies, suggests that DNA cross-links are responsible for the embryo toxic effects of CPA (Little and Mirkes, 1987). The study reported that not only the G-NOR-G adduct but also the mono adducts NOR-G and N-[2-(N7-guaninyl) ethyl]-N-[2-
hydroxyethyl]-amine (NOR-G-OH) were formed in calf thymus DNA alkylated in vitro with PM using tandem mass spectrometric parent ion scanning (Cushnir et al., 1990).

Previous studies reported that ~90% of CPA-induced DNA lesions were guanine mono adducts (Hemminki, 1985; Souliotis et al., 2003) and that the emergence of such adducts correlated with the onset of cytotoxicity (Benson et al., 1988). However, 1,3-interstrand G-NOR-G lesions are also thought to play a central role in the cytotoxic response because they prevent DNA strand separation which is required for DNA replication and transcription (Rajski and Williams, 2000; Souliotis et al., 2003). Therefore, G-NOR-G lesions are considered the primary cytotoxic adducts responsible for the antineoplastic activity of CPA in vivo (Hemminki, 1985; Benson et al., 1988). PM destroys rapidly dividing cells including cancer cells by covalently binding to DNA, inducing DNA-DNA, DNA-protein cross links and DNA double strand breaks (DSB) (Helleday et al., 2008; Johnson et al., 2011) (Figure 6).

PM is also thought to be responsible for the ovarian follicle destruction caused by CPA (Plowchalk and Mattison, 1991; Anderson et al., 1995; Desmeules and Devine, 2006; Petrillo et al., 2011). The ovary does not appear to bioactivate CPA, rather CPA bioactivation is thought to occur in the liver by cytochrome P450 enzymes with uptake of the reactive metabolites from the blood (Anderson et al., 1995; Colvin, 1999). PM is the ultimate ovotoxicant of CPA (Plowchalk and Mattison, 1991) in that it can, with only a brief exposure, induce rapid loss of dormant and small preantral follicles at concentrations relevant to those found in the blood of human patients (Struck et al., 1987). The cellular target of PM appears to depend on the follicle type, with oocytes being affected in smaller follicles and granulosa cells being predominantly affected in larger growing follicles (Desmeules and
Devine, 2006). PM causes dose- and time-dependent primordial and primary ovarian follicle loss in mice and rat oocytes (Petrillo et al., 2011). Also, an in vitro study demonstrating that a volatile compound can induce primordial follicle loss suggests this breakdown product may be also toxic to the ovary (Madden et al., 2014). Flowers et al. (2000) provided strong evidence that this chemical is chloroethylaziridine (CEZ) presented in cultured human breast tumor cell lines MCF-7wt (wild type) and MCF-7hc (4-HC resistant subline). Such effects suggest that CEZ could contribute significantly to CPA-induced ovarian toxicity in vivo, since concentrations in those wells in vitro are likely much less than that of PM in wells (i.e., <10 µM). CEZ is volatile as well as cytotoxic and is liberated through alkylation reactions with nucleophiles and P-N bond hydrolysis from PM (Shulman-Roskes et al., 1998). The generated CEZ was very stable in the culture medium with a degradation half-life of 265 h. The distribution of products from PM as well as the lifetime of CEZ itself is very dependent upon the nature and concentration of nucleophiles in solution (Shulman-Roskes et al., 1998). Nevertheless, CEZ must be considered an alternative (or additional) source of ovarian toxicity (Flowers et al., 2000; Madden et al., 2014).

Polycyclic aromatic hydrocarbons (PAHs) are atmospheric pollutants that consist of fused aromatic rings, and human exposure arises from byproducts of organic matter burning. PAH’s have carcinogenic, mutagenic and teratogenic effects (Shimada, 2006). DMBA is a PAH liberated from burning of organic matter, cigarette smoke and car exhaust fumes. Cigarette smoking accelerates entry of females into the post-menopausal physiological state (Jick and Porter, 1977) and contains high level of the PAH’s DMBA, BaP and 3-MC (Mattison et al., 1983). In vivo exposure of mice and rats has demonstrated that DMBA depletes primordial follicles (Mattison and Schulman, 1980; Borman et al., 2000) and
induces ovarian tumor formation (Krarup, 1967; Krarup, 1969). Mice treated with DMBA (80 mg/kg) also showed signs of necrosis in primordial follicles (Mattison and Nightingale, 1980). DMBA destroys all ovarian follicle types in mice and rats in a dose-dependent manner (Matikainen et al., 2001; Rajapaksa et al., 2007; Igawa et al., 2009), and follicle destruction is initiated by oocyte loss followed by somatic cell apoptosis (Morita and Tilly, 1999). Due to the loss of all follicle types, a reduction in ovarian volume typically occurs (Mattison and Schulman, 1980; Weitzman et al., 1992). DMBA causes apoptosis in granulosa and theca cells of pre-ovulatory follicles through increased expression of pro-apoptotic BAX and activation of the executioner protein caspase 3 (CASP3) (Tsai-Turton et al., 2007). PAH’s require metabolism to produce reactive ovotoxic metabolites (Mattison et al., 1983) that bind to cellular macromolecules including DNA, RNA and protein (Sims and Grover, 1974). In hepatic tissue, DMBA undergoes extensive metabolism to form bioactive metabolites (Bengtsson et al., 1983). The metabolism pathway begins with DMBA oxidation to DMBA-3,4-diol by CYP 1B1 and hydrolysis by microsomal epoxide hydrolase (mEH). The 3,4-diol metabolite undergoes further epoxidation by CYP1A1 and CYP1B1 to form the ultimate ovotoxicant and carcinogen, DMBA-3,4-diol-1,2 epoxide (Miyata et al., 1999; Kleiner et al., 2004; Shimada and Fujii-Kuriyama, 2004) (Figure 7). Utilizing a whole ovary culture model to study metabolism of DMBA has further supported the capacity of the ovary for bioactivation of DMBA (Igawa et al., 2009). Cultured rat ovaries were exposed to DMBA or DMBA-3,4-diol at concentrations ranging from 12.5 to 1000 nM for 15 days. Primordial follicle loss occurred at a concentration of 75 nM DMBA and 12.5 nM DMBA-3,4-diol. Small primary follicle loss followed a similar pattern, with follicle destruction occurring at 375 nM DMBA and 75 nM DMBA 3,4-diol (Igawa et al., 2009). Therefore, the lower
concentrations required to cause follicle loss indicate that the 3,4-diol metabolite is more ovotoxic than the DMBA parent compound. These metabolites are capable of covalent binding to macromolecules such as DNA, RNA and protein to form adducts. DMBA-3,4-diol-1,2-epoxide binds to DNA to form DNA adduct in female rat mammary tissue which ultimately leads to DNA damage (Singletary et al., 1996). DMBA can cause dose-dependent DNA damage in extra-ovarian tissues (peripheral lymphocytes, liver and skin cells) when exposed along with physical stress in rats (Muqbil et al., 2006).

**DNA Damage Responses**

The maintenance of genome integrity and fidelity is essential for the proper function and survival of all organisms. This task is particularly daunting due to constant assault on the DNA by genotoxic agents (both endogenous and exogenous) (Lindahl, 1993). DNA damage is an alteration in the chemical structure of DNA, such as a break in a strand of DNA, a base missing from the backbone of DNA, or a chemically changed base such as 8-OHdG. Failure to repair DNA lesions may result in blockages of transcription and replication, mutagenesis, and/or cellular cytotoxicity. In humans, DNA damage has been shown to be involved in a variety of genetically inherited disorders, in aging (Finkel and Holbrook, 2000) and in carcinogenesis (Hoeijmakers, 2001). All eukaryotic cells have evolved a multifaceted response to counteract the potentially deleterious effects of DNA damage. Upon sensing DNA damage or stalls in replication, cell cycle checkpoints are activated to arrest cell cycle progression to allow time for repair before the damage is passed on to daughter cells. In addition to checkpoint activation, the DNA damage response leads to induction of transcriptional programs, enhancement of DNA repair pathways, and when the level of
damage is severe, to initiation of apoptosis (Zhou and Elledge, 2000; Friedberg, 2003) (Figure 8). All of these processes are carefully coordinated so that the genetic material is faithfully maintained, duplicated, and segregated within the cell. Cell cycle checkpoints are regulatory pathways that govern the order and timing of cell cycle transitions to ensure completion of one cellular event prior to commencement of another.

Cell Cycle Arrest

The key regulators of the checkpoint pathways in the mammalian DNA damage response are the ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) protein kinases. ATM is the primary mediator of the response to DNA double strand breaks (DSBs) that can arise by exposure to ionizing radiation (IR), chemotherapeutic drug and other environmental exposures. Central to this checkpoint is the accumulation and activation of the P53 protein; two properties carefully controlled by the ATM and ATR kinases. Following IR damage, ATM activates downstream kinase check point kinase 2 (CHK2) (by phosphorylation at position T68) (Matsuoka et al., 2000) which in turn phosphorylates a residue of P53. The phosphorylation of P53 blocks P53/MDM2 interaction, resulting in P53 accumulation. The residue of P53 can be phosphorylated directly by ATM or ATR in response to IR (ATM and ATR), UV irradiation (ATR) and stalls of DNA replication forks (ATR). Activated P53 then up-regulates a number of target genes, several of which are also involved in the DNA damage response (MDM2, GADD45a, and P21). The accumulation of P21 (cyclin-dependent kinase inhibitor) suppresses CCNE1/CDK2 kinase activity thereby resulting in G1 arrest (Bartek and Lukas, 2001) (Figure 9). Upon IR damage, ATM phosphorylates a number of downstream substrates including Nijmegen breakage syndrome
(NBS1), the product of the breast cancer susceptibility gene 1 (BRCA1), and structural maintenance of chromosome protein 1 (SMC1). Loss of any of these proteins or mutation of the indicated phosphorylation sites results in attenuated S-phase checkpoint activation (Kim et al., 2002). Upon DNA damage, the downstream kinases CHK1 and CHK2 phosphorylate CDC25C on position S216. Phosphorylation of this residue creates a binding site for the 14-3-3 proteins. The 14-3-3/CDC25C protein complexes are sequestered in the cytoplasm, thereby preventing CDC25C from activating CDC2 through removal of the T14 and Y15 inhibitory phosphorylation. This results in the maintenance of the CDC2/CCNB1 complex in its inactive state and blockage of entry into mitosis (Peng et al., 1997).

**DNA Repair Pathways**

The simplest of the human DNA repair pathways involves the direct reversal of the highly mutagenic alkylation lesion O6-methylguanine (O6-mG) by the product of the O6-methylguanine DNA methyltransferase (Mgmt) gene (Margison and Santibáñez-Koref, 2002). Base excision repair (BER) is a multi-step process that corrects non-bulky damage to bases resulting from oxidation, methylation, deamination, or spontaneous loss of the DNA base itself. These alterations, although simple in nature, are highly mutagenic and therefore represent a significant threat to genome fidelity and stability. Nucleotide excision repair (NER) is perhaps the most flexible of the DNA repair pathways considering the diversity of DNA lesions it acts upon. The most significant of these lesions are pyrimidine dimers (cyclobutane pyrimidine dimers and 6-4 photoproducts) caused by the UV component of sunlight. Other NER substrates include bulky chemical adducts, DNA intra-strand crosslinks, and some forms of oxidative damage. The common features of lesions recognized by the
NER pathway are that they cause both a helical distortion of the DNA duplex and a modification of the DNA chemistry (Buschta-Hedayat et al., 1999). Heterotrimeric replication protein A (RPA) binds at the site of injury to aid in damage recognition. The DNA mismatch repair (MMR) pathway plays an essential role in the correction of replication errors such as base-base mismatches and insertion/deletion loops (IDLs) that result from DNA polymerase misincorporation of nucleotides and template slippage, respectively.

DNA DSBs are perhaps the most serious form of DNA damage because they pose problems for transcription, replication, and chromosome segregation. Damage of this type is caused by a variety of sources including exogenous agents such as IR and certain genotoxic chemicals, endogenously generated reactive oxygen species, replication of single-stranded DNA breaks, and mechanical stress on the chromosomes. DSBs differ from most other types of DNA lesions in that they affect both strands of the DNA duplex and therefore prevent use of the complementary strand as a template for repair. Failure to repair these defects can result in chromosomal instabilities leading to dysregulated gene expression and carcinogenesis (Hoeijmakers, 2001). DSBs and stalled replication forks cause the phosphorylation of neighboring histone 2AX at Ser139 (γH2AX) via ATM and ATR respectively. For DSBs, the distribution of γH2AX within the nucleus is localized around the DSB and these γH2AX foci appear approximately 10 min after DSB induction (Rogakou et al., 1998). Over time the amount and intensity of these γH2AX foci reaches a maximum (at about 1 h) and later on decreases due to the activity of the phosphatase PP4 showing that DSB repair has occurred (Nakada et al., 2008). As stated above, stalled replication forks activate ATR which phosphorylates H2AX. The functional significance of γH2AX is assumed to be a signal that facilitates the repair of free DSBs or DSBs that are formed at stalled replication forks,
presumably by causing the chromatin to be more accessible for DNA repair. To counteract
the detrimental effects of these potent lesions, cells have evolved two distinct pathways of
DSB repair, homologous recombination (HR) and non-homologous end joining (NHEJ)
(Jackson, 2002) (Figure 10). The cellular decision, to which pathway to utilize for DSB
repair is unclear, however, it appears to be largely influenced by stage within the cell cycle at
the time of damage acquisition (Takata et al., 1998).

HR-directed repair corrects DSB defects in an error-free manner using a mechanism
that retrieves genetic information from a homologous, undamaged DNA molecule. The
majority of HR-based repair takes place in late S- and G2-phases of the cell cycle when an
undamaged sister chromatid is available for use as repair template. The RAD52 epistasis

group of proteins, including RAD50, RAD51, RAD52, RAD54, and MRE11 mediate this

process (Sonoda et al., 2001). The RAD52 protein itself is thought to be the initial sensor of
the broken DNA ends. Processing of the damaged ends ensues resulting in the production of
3’ single-stranded DNA (ssDNA) overhangs. The newly generated ssDNA ends are bound by
RAD51 to form a nucleoprotein filament. Other proteins including RPA, RAD52, RAD54,
BRCA1, BRCA2, and several additional RAD51-related proteins serve as accessory factors

in filament assembly and subsequent RAD51 activities (Jackson, 2002). The RAD51
nucleoprotein filament then searches the undamaged DNA on the sister chromatid for a
homologous repair template. Once the homologous DNA has been identified, the damaged
DNA strand invades the undamaged DNA duplex in a process referred to as DNA strand
exchange. A DNA polymerase then extends the 3’ end of the invading strand and subsequent
ligation by DNA Ligase I yields a heteroduplexed DNA structure. This recombination
intermediate is resolved and the precise, error-free correction of the DSB is complete. In
contrast to HR, NHEJ does not require a homologous template for DSB repair and usually results in the correction of the break in an error-prone manner. Essential to the NHEJ pathway is the activity of the KU70/KU80 heterodimeric protein (Featherstone and Jackson, 1999). The KU heterodimer initiates NHEJ by binding to the free DNA ends and recruiting other NHEJ factors such as DNA-dependent protein kinase (DNA-PK), XRCC4, and DNA Ligase IV to the site of injury (Jackson, 2002). DNA-PK becomes activated upon DNA binding, and phosphorylates a number of substrates including P53, KU, and the DNA Ligase IV cofactor XRCC4. Phosphorylation of these factors is believed to further facilitate the repair process. Because the ends of most DSBs generated by genotoxic agents are damaged and unable to be directly ligated, they often have to undergo limited processing by nucleases and/or polymerases before NHEJ can proceed. The nuclease(s) responsible for this activity, include MRE11/Rad50/NBS1 complex, FEN-1(Haber, 1998; Wu et al., 1999; Petrini, 2000), and the Artemis protein (Moshous et al., 2001). The final step in NHEJ repair involves ligation of the DNA ends by Ligase IV in a complex that also includes XRCC4 and KU. If DNA repair fails, cells undergo death by activating an apoptotic pathway.

**Apoptosis**

The integrity of genomic DNA is constantly under threat, even in perfectly healthy cells. DNA damage can result from the action of endogenous reactive oxygen species or from stochastic errors in replication or recombination, as well as from environmental and therapeutic genotoxins. The current paradigm states that if DNA repair fails, cells undergo death by activating one of the programmed death pathways, i.e. apoptosis. This implies that cells containing sufficient DNA damage to overwhelm its capability of repairing its DNA
will be eliminated from the population in a given tissue. Induction of apoptosis has been recognized as a possible outcome of DNA damage for more than 20 years (Wyllie et al., 1980). There are various experimental systems, which show that defects in the execution of apoptosis increase cancer incidence (Wirtz et al., 2010). Thus apoptosis following DNA damage is a protective mechanism that prevents carcinogenesis.

Apoptosis is a process of programmed cell death that occurs during DNA damage in some cells. The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Elmore, 2007) (Figure 11). However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of CASP3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway resultant from single stranded DNA damage (Martinvalet et al., 2005).

Tumor cells and virus-infected cells are killed by perforin/granzyme pathways where secretion of the transmembrane pore-forming molecule perforin and exophytic release of cytoplasmic granules into the target cells occur (Trapani and Smyth, 2002). The serine
proteases granzyme A and granzyme B are the most important component within the granules (Pardo et al., 2004). Granzyme B uses both the mitochondrial pathway and direct activation of CASP3 to kill cells (Goping et al., 2003). Granzyme A is also important in cytotoxic T cell induced apoptosis and activates caspase independent pathways. Inactivation of this complex (SET, Ape1, pp32, and HMG2) by granzyme A most likely contributes to apoptosis by blocking the maintenance of DNA and chromatin structure integrity (Lieberman and Fan, 2003).

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). The binding of Fas ligand to Fas receptor results in the binding of the adapter protein, Fas-associated protein with death domain (FADD) and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein, Tumor necrosis factor receptor type 1-associated death domain (TRADD) with recruitment of FADD and RIP (Hsu et al., 1995; Wajant, 2002). FADD then associates with pro-CASP8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of pro-CASP8 (Kischkel et al., 1995). Once CASP8 is activated, the execution phase of apoptosis is triggered.

The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway include but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All of these stimuli cause changes in the inner mitochondrial membrane that
results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of cytochrome c (CYCS) from the intermembrane space into the cytosol (Saelens et al., 2004). CYCS binds and activates Apaf-1 as well as pro-CASP9, forming an apoptosome (Chinnaiyan, 1999; Hill et al., 2004). The clustering of pro-CASP9 in this manner leads to CASP9 activation. The control and regulation of these apoptotic mitochondrial events occurs through members of the BCL-2 family of proteins (Cory and Adams, 2002), regulate mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include BCL-2, BCL-X, BCL-XL, BCL-XS, BCL-W, BAG, and some of the pro-apoptotic proteins include BCL-10, BAX, BAK, BID, BAD, BIM, BIK, and BLK. The BCL-2 and BCL-XL inhibit apoptotic death primarily by controlling the activation of caspase proteases (Newmeyer et al., 2000). P53 up regulated modulator of apoptosis (PUMA) and Phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) are two members of the BCL2 family that are also involved in pro-apoptosis. PUMA plays an important role in P53-mediated apoptosis. It was shown that, in vitro, overexpression of PUMA is accompanied by increased BCL2-associated X protein (BAX) expression, BAX conformational change, translocation to the mitochondria, CYCS release and reduction in the mitochondrial membrane potential (Liu et al., 2003). PMAIP1 is also a candidate mediator of P53-induced apoptosis. Studies show that this protein can localize to the mitochondria and interact with anti-apoptotic BCL-2 family members, resulting in the activation of CASP9 (Oda et al., 2000). Since both PUMA and PMAIP1 are induced by P53, they might mediate the apoptosis that is elicited by genotoxic damage or oncogene activation.
The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. It is the activation of the execution CASP that begins this phase of apoptosis. Execution CASP activates cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. CASP3, CASP6, and CASP7 function as effector or executioner CASP, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NUMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Slee et al., 2001). CASP3 is considered to be the most important of the executioner CASP and is activated by any of the initiator CASP. In apoptotic cells, activated CASP3 cleaves inhibitor of CASP-activated DNase (ICAD) to release CASP-activated DNase (CAD) (Sakahira et al., 1998). CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. CASP3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies.

**P53-Independent Apoptotic Pathway**

As mentioned above, DSBs activate ATM resulting in phosphorylation of P53. Upon phosphorylation, P53 blocks proliferation by upregulation of P21, which triggers G1-S arrest at low level of DSBs (Roos and Kaina, 2006). With higher level of DSBs, P53 accumulates and activate pro-apoptotic genes such as BAX and PUMA (Lane, 1992). Atm mutated cells showed low level of apoptosis than normal cells following irradiation exposure (Sengupta et al., 2013), indicating that ATM-P53 pathway is crucial for DNA damage-induced apoptosis. Fibroblasts that express ATM are less sensitive to apoptosis induced by alkylating agents than Atm knockout fibroblast in a P53-mutated background (Debiak et al., 2004).
suggests that DNA damage-induced apoptosis are not only triggered by ATM-P53 signaling, it can also be triggered by P53-independent pathway (Figure 12) (Roos and Kaina, 2006). There are several strategies that cells employ to trigger P53-independent DNA damage-induced apoptosis. The first one involves the P53 homologs P63 and P73 (Melino et al., 2002). It has been proposed that activation of ATM and/or ATR by DNA damage recruit CHK1 and CHK2 to activate E2F1 by etoposide or camptothecin exposure (Urist et al., 2004). E2F1 is a transcription factor that has unique ability to induce apoptosis. Chemotherapeutic drugs such as the topoisomerase II inhibitors, etoposide and adriamycin causes apoptosis through overexpression of E2F1 (Nip et al., 1997; Pruschy et al., 1999). The role of E2F1 is proposed by its over expression after DNA damage in the response to DNA damage (Blattner et al., 1999) by an ATM-induced phosphorylation (Lin et al., 2001). This in turn stimulates transcription of the p73 gene, giving rise to an increased level of P73 protein (Urist et al., 2004). P53 requires P63 and P73 for triggering apoptosis (Flores et al., 2002). However, P73 is pro-apoptotic even in the absence of P53. Up regulation of P73 expression by p53 inactivation through E2F1 mediated transcription in human breast cancer cells (Tophkhane et al., 2012). P73-induced apoptosis was shown to be mediated by transcriptional upregulation of PUMA, which in turn provokes BAX mitochondrial translocation and CYCS release (Melino et al., 2004). P73 was also shown to activate the P53 target gene that encodes PMAIP1, which causes mitochondrial dysfunction (Flinterman et al., 2005).

Voltage-dependent anion channel (VDAC) is present at the external mitochondrial membrane, for transporting anions, cations, ATP, Ca^{2+} and metabolites between mitochondria and other parts of the cell (Keinan et al.). It is well established that VDAC is a
major permeability pathway for the transfer of Ca\(^{2+}\) across the outer mitochondrial membrane (Kroemer et al., 2008; Shelby, 2008). VDAC1 regulates the release of apoptotic proteins from mitochondria such as CYCS by oligomerization (Igosheva et al., 2010). The release of CYCS is also regulated by BCL-2 family protein BAX, which interacts with VDAC to increase pore size and promote CYCS release, while anti-apoptotic BCL-XL produces the exact opposite effect (Wu et al., 2010b). Chemotherapeutic agents interfere with BCL-XL mitochondrial binding by VDAC1-based peptides to induce apoptosis in cancer cells (Arbel et al., 2012). This key role in apoptosis suggests VDAC as a potential target for chemotherapeutic drugs.

**Summary**

This literature review indicates that maintenance of ovarian follicular reserve is critical for normal reproductive function. Ovarian follicular viability and endowment are not only affected by chemical exposure but also obesity. Ovotoxicants can be bioactivated in ovarian tissue via CYP and mEH enzymes and interact with DNA to form DNA adducts, ultimately leading to DNA damage which is a potential upstream event of the chemical-induced ovarian follicle loss. The purpose of the studies described in this dissertation was to elucidate upstream mechanistic initiating events leading to ovotoxicity induced by PM and DMBA. In addition, the impact of altered central metabolism on ovarian susceptibility to the ovotoxic effects of PM and DMBA were evaluated. Our rationale for these studies was that these two ovotoxicants have been demonstrated to interact with DNA in extra-ovarian tissues. Additionally, ovaries from a progressive model of obesity had increased sensitivity to DMBA-induced ovotoxicity, and had basal levels of DNA damage (Nteeba et al., 2014).
Three models were used to assess the impacts of PM and DMBA exposure on DNA damage, DNA repair, GJ communication, and altered signaling pathways; a spontaneously immortalized granulosa cell line; use of a neonatal whole ovary culture system; as well as in vivo studies using the lethal yellow mouse model of progressive obesity.

References


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Folliculogenesis begins with the establishment of a finite pool of primordial follicles. Primordial follicles must grow to the primary, pre-antral and antral stages before they reach the pre-ovulatory stage and are capable of releasing an oocyte for fertilization. After ovulation, the remaining granulosa and theca cells differentiate into a structure known as the corpus luteum (CL). At the antral stage, however, 99.9% of antral follicles will undergo atresia (Barnett et al., 2006).
Figure 2. The arrangement of gap junction

Each gap junction is composed of two connexins contributed by each cell. Individual connexins are made up of six protein subunits called connexons. Each connexin protein contains amino and carboxyl terminus in the cytoplasm. Four transmembrane domains in connexin are connected by two extracellular loops (E1 and E2) and one cytoplasmic loop (CL) (Tripathi, P and Tripathi, M; 2010).
Figure 3. Formation of hemichannels and intercellular channels

(A) Six identical connexin subunits (green or orange) can oligomerize to form a homomeric hemichannel. Two co-expressed connexins may oligomerize with each other to form a heteromeric hemichannel. (B) Two hemichannels dock with each other to form a complete gap junction channel. Two hemichannels of similar composition form homotypic channels whereas two hemichannels of different composition form heterotypic channels (Beyer et al., 2013).
Figure 4. The potential effects of reproductive toxicants on the reproductive life

Exposure to reproductive toxicants can have long term impact on female reproductive capacity. The effect can vary depending upon whether the damage occurred during fetal development, childhood, regular adulthood or pregnancy. One possible result can be premature ovarian failure (menopause) (Hoyer P.B., Comprehensive Toxicology, Vol. 10, Elsevier Publishing, Oxford, 1997, p 253).
Figure 5. The human ovary and potential targets of chemotherapeutic agents

A) Stages of follicle development and B) Potential targets of chemotherapeutic damage within the ovary (i) chemotherapeutic agents could be directly affecting the resting pool of primordial follicles or the growing follicle population. As growing follicles inhibit the recruitment of primordial follicles, the loss of this growing population will lead to increased activation of primordial follicles and so the loss of that reserve. (ii) Chemotherapeutic agents could be directly targeting the oocyte or the somatic cells. Oocyte death would result from death of the follicular somatic cells, as the oocyte is dependent on these for its survival (Morgan et al., 2012).
Cyclophosphamide converted into acrolein and phosphoramide mustard with help of liver microsomes. Phosphoramide mustard bind with DNA at guanine position to form G-NOR, G-NOR-OH and G-NOR-G adducts (Johnson et al., 2011).

Figure 6. Cyclophosphamide metabolism and formation of DNA adducts
Figure 7. DMBA metabolic pathway

The parent compound, DMBA, is bioactivated by CYP450 isoform 1B1 (CYP1B1) to a DMBA-3, 4-epoxide intermediate, which is hydrolyzed by mEH to form DMBA-3,4-diol. This compound further undergoes bioactivation by either CYP1B1 or 1A1 to form the ultimate carcinogenic and ovotoxic metabolite, DMBA-3,4-diol-1,2-epoxide (Miyata et al., 1999).
Figure 8. DNA damage responses

DNA damage is caused by a variety of sources. The cellular response to damage may involve activation of a cell cycle checkpoint, commencement of transcriptional programs, execution of DNA repair, or when the damage is severe, initiation of apoptosis (Friedberg et al., 1995).
Figure 9. Mammalian cell cycle checkpoint pathways

In response to DNA damage, ATM and/or ATR trigger the activation of a checkpoint that leads to cell cycle arrest or delay. Checkpoint pathways are characterized by cascades of protein phosphorylation events (indicated with a "P") that alter the activity, stability, or localization of the modified proteins. A general overview of the G1, S, and G2 cell cycle checkpoint pathways is indicated (Bartek, J. & J. Lukas 2001).
Figure 10. Double strand break (DSB) repair

Shown is an overview of the main steps and factor requirements for DNA DSB repair by homologous recombination (left) and non-homologous end-joining (right) (Jackson, S.P. 2002).
Figure 11. Schematic representation of apoptotic events

The two main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/granzyme pathway. Each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathways activates its own initiator caspase (8,9,10) which in turn will activate the executioner caspase-3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages (Elmore, 2007).
Figure 12. Mechanism of P53-independent apoptosis

DNA damage activates ATM or ATR, and this activates CHK1 or CHK2. CHK1 and CHK2 activate E2F1 and the transcription factor E2F1 transcribes the P73 protein. P73 mediates apoptosis through either the transcription of NOXA, PUMA and BAX or via mitochondrial localization of these proteins (Roos et al., 2000).
CHAPTER 2. 7,12-DIMETHYLBENZ[A]ANTHRACENE EXPOSURE INDUCES THE DNA REPAIR RESPONSE IN NEONATAL RAT OVARIES


Shanthi Ganesan, Poulomi Bhattacharya and Aileen F. Keating

Contribution Statement:

I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Poulomi Bhattacharya performed the γH2AX western blot. Aileen F. Keating designed experiments, aided in data interpretation and edited the manuscript.

Abstract

7,12-dimethylbenz[a]anthracene (DMBA) destroys ovarian follicles at all stages of development. This study investigated DMBA-induced DNA double strand break (DSB) formation with subsequent activation of the ovarian DNA repair response in models of pre-antral or pre-ovulatory follicle loss. Postnatal day (PND) 4 Fisher 344 (F344) rat ovaries were cultured for 4 days followed by single exposures of vehicle control (1% DMSO) or DMBA (12.5 nM or 75 nM) and maintained in culture for 4 or 8 days. Alternately, PND4 F344 rat ovaries were exposed to 1 µM DMBA at the start of culture for 2 days. Total RNA or protein was isolated, followed by RT-PCR or Western blotting to quantify mRNA or protein level, respectively. γH2AX and phosphorylated ATM were localized and quantified using immunofluorescence staining. DMBA exposure increased CASP 3 and γH2AX protein. Additionally, DMBA (12.5 nM and 1 µM) increased levels of mRNA encoding Atm, Xrcc6, Brca1 and Rad51. In contrast, Parp1 mRNA was initially decreased (d4) but subsequently increased (d8), due to DMBA, and PARP1 protein was increased after 8 days of exposure. Total ATM increased in a concentration-dependent temporal pattern (75 nM d4; 12.5 nM d8), while pATM was localized in large primary and secondary follicles and increased after 8
days of 75 nM DMBA exposure compared to both control and 12.5 nM DMBA. These findings support that, despite some concentration effects, DMBA induces ovarian DNA damage and that DNA repair mechanisms are induced as a potential mechanism to prevent follicle loss.

**Introduction**

The ovary is the ovum-producing female reproductive organ composed of follicles at different stages of development. The oocytes encased in primordial follicles are maintained in a dormant state until activation into the growing follicular pool where they subsequently go through a series of follicular developmental stages (McGee and Hsueh, 2000). Approximately 99% of ovarian follicles die by a process known as atresia (Borman *et al.*, 2000), and ovarian senescence (menopause) occurs when the finite pool of primordial follicles has become exhausted (Hoyer and Sipes, 1996; Broekmans *et al.*, 2007; Hansen *et al.*, 2008). Since primordial follicles cannot be regenerated (Hirshfield, 1991), their depletion leads to infertility and premature ovarian failure (POF). A number of chemical classes can deplete follicles causing ovotoxicity, including the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA) (Mattison, 1980; Hoyer *et al.*, 2009; Igawa *et al.*, 2009).

DMBA causes destruction of all follicle types leading to POF in mice and rats (Mattison, 1980). Sources of environmental DMBA exposure come from burning of organic matter, including cigarette smoke, charred foods and car exhaust fumes (Gelboin, 1980). DMBA causes apoptosis in granulosa and theca cells of pre-ovulatory follicles through increased expression of pro-apoptotic BAX and activation of the executioner protein CASP3
(Tsai-Turton et al., 2007). Cigarette smoke exposure induces early menopause in female smokers compared to their non-smoking counterparts (Jick, 1979; Mattison et al., 1983; Harlow and Signorello, 2000). Also, the offspring of female smokers, are born with a decreased number of oocytes, potentially leading to abnormal ovarian function, decreased fertility, and/or early menopause, even if smoking was halted during pregnancy (Jurisicova et al., 2007). Chronic low dose DMBA exposure (0.35 mg/kg to 7.0 mg/kg intraperitoneal (i.p) daily for 15d), mimicking human DMBA exposure, resulted in more follicle loss compared to a single high dose (80mg/kg i.p) in rats (Borman et al., 2000). Use of a neonatal ovary culture system has determined that a single DMBA exposure depleted large primary follicles at concentrations of 12.5 and 75 nM, while secondary follicles were destroyed at the 12.5 nM concentration only (Madden et al., under review), further suggesting that single low dose DMBA exposures are of equal concern as high dose exposures for ovarian function.

DMBA can cause dose-dependent DNA damage in extra-ovarian tissues (peripheral lymphocytes, liver and skin cells) when exposed along with physical stress in rats (Muqbil et al., 2006). Double-strand breaks (DSB) in DNA are the most cytotoxic lesions, generated by ionizing radiation, man-made chemicals (van Gent et al., 2001) and chemotherapeutic drugs (Helleday et al., 2008). These DSB’s pose a serious threat to genome stability if either unrepaired or repaired incorrectly, and could potentially lead to permanent damage that could be a negative consequence for gamete health (Petrillo et al., 2011; Summers et al., 2011). DSB’s can be sensed by Ataxia telangiectasia mutated (ATM) protein, a phosphatidylinositol-3 kinase (PI3K) family member, with subsequent activation of cellular DNA damage responses (DDR) leading to cell cycle arrest, DNA damage repair, and subsequent cell cycle resumption (Norbury and Hickson, 2001; Shiloh, 2003; Yang et al.,
2003; Giunta et al., 2010). One event downstream of ATM activation is phosphorylation of histone H2AX (termed γH2AX) which leads to recruitment of DNA repair molecules to the site of damage (Svetlova et al., 2010).

There are two major pathways that repair DNA DSB’s: Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ). HR requires regions of extensive DNA homology and is most active in the S and G2 phases of the cell cycle in dividing cells (Chiruvella et al., 2012). During HR, breast cancer type 1 (BRCA1) is phosphorylated by ATM and co-localizes with RAD51 at the site of DNA damage to induce DSB repair (Scully et al., 1997). NHEJ, active at all phases of the cell cycle, can rejoin a DSB with or without processing of the ends (Chiruvella et al., 2012). Key signaling molecules involved in NHEJ repair include the KU70/80 heterodimer (XRCC6 and XRCC5) which recognize and bind to the DSB, recruiting DNA dependent-protein kinase (DNA-PKs) to the DSB ends (Calsou et al., 2003). In addition, Artemis, X-ray repair complementing defective repair in Chinese hamster cells 4 (XRCC4), DNA ligase IV, and XRCC4-like factor (XLF) are activated during NHEJ repair (Dobbs et al., 2010).

Little is known about whether DNA damage is induced in the ovary during DMBA exposure, and if so, which DNA repair pathway predominates. The objective of this study was therefore to investigate the DNA repair response induced by DMBA at concentrations that induce pre-antral (primordial and small primary; 1 µM DMBA) and pre-ovulatory (large primary and secondary; 12.5 and 75 nM DMBA) follicle depletion.
Methods and Materials

Reagents

7,12-Dimethylbenz[a]anthracene (DMBA; CAS # 57-97-6), bovine serum albumin (BSA), ascorbic acid, transferrin, 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N’N’N’-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, Sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco's Modified Eagle Medium:nutrient mixture F-12 (Ham) 1x (DMEM/Ham's F12), Albumax, penicillin (5000U/ml), Hanks' Balanced Salt Solution (without CaCl₂, MgCl₂ or MgSO₄) from Invitrogen Co. (Carlsbad, CA). Millicell-CM filter inserts and 48 well cell culture plates were obtained from Millipore (Bedford, MA) and Corning Inc. (Corning, NY) respectively. RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-mouse, goat anti-rabbit and rabbit anti-goat secondary antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

Ovary culture

Ovaries were collected from PND4 female F344 rats and cultured as described previously (Devine et al., 2002). Briefly, ovaries were removed, trimmed of oviduct and other excess tissues and placed onto membrane floating on 250 μl of DMEM/Ham's F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 μg/ml ascorbic acid, 5 U/ml
penicillin and 27.5 μg/ml transferrin per well in a 48 well plate that had previously been equilibrated to 37 °C. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries were cultured in control medium for four days to allow development of large primary and secondary follicles, and were then treated once with medium containing vehicle control (1% DMSO) ± DMBA (12.5 nM or 75 nM) and the culture was maintained for four or eight days (as described below) at 37 °C and 5% CO₂. This exposure induces pre-ovulatory follicle depletion after 8 days (Madden et al., under review). In addition, ovaries were exposed to vehicle control (1% DMSO) ± DMBA (1 μM) on alternate days from the start of culture (PND4) for 2 days. At this concentration, pre-antral follicle loss occurs from four days onward (Igawa et al., 2009). The medium was replaced every two days. One ovary per animal was placed in control medium, while the contralateral ovary was exposed to the experimental treatment. All animal procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University.

**RNA isolation and qRT-PCR**

RNA was isolated using an RNeasy Mini kit (Qiagen) and the concentration was determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE) (n=3; 10 ovaries per pool). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). cDNA was diluted (1:20) in RNase-free water. Diluted cDNA (2μl) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers for *Atm, Brca1, Parp1, Rad51, Xrcc6* and *Gapdh* were designed by Primer 3 Input Version (0.4.0) and are listed in Table 1. The regular cycling program consisted of a 15-min hold at 95°C and 45 cycles of denaturing at 95°C for 15s, annealing at 58°C for 15s, and extension at 72°C for 20s
at which point data were acquired. There was no difference in \textit{Gapdh} mRNA expression between treatments, thus each sample was normalized to \textit{Gapdh} before quantification. Quantification of fold-change in gene expression was performed using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

\textbf{Protein isolation and Western blotting}

Protein was isolated from cultured ovaries (n=3; 10 ovaries per pool). Homogenates were prepared from cultured ovaries via homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Thompson \textit{et al.}, 2005). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a BCA protocol. Protein was stored at -80 °C until further use. SDS-PAGE was used to separate protein homogenates which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 5% milk in Tris-buffered saline containing tween 20, followed by incubation with anti-PARP1 antibody (1:200), anti-ATM antibody (1:100), anti-RAD51 antibody (1:500), anti-$\gamma$H2AX (1:200) or anti-caspase 3 antibody (1:50) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with species-specific secondary antibodies (1:2000) for 1 h at room temperature. Membranes were washed 3X in TTBS and incubated in chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values.
**Immunofluorescence Staining**

Ovaries were fixed in 4% paraformaldehyde for 2 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 µM thick), and every 10th section was mounted. Slides were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1M, pH 6.1). Sections were then blocked in 5% BSA for 1 h at room temperature. Sections were incubated with primary antibodies directed against pATM (1:100) or γH2AX (1:50) overnight at 4°C. After washing in 1% PBS, sections were incubated with the appropriate goat anti-mouse IgG-FITC or donkey anti-rabbit IgG-FITC secondary antibodies for 1 h. Slides were then counterstained with 4-6-diamidino-2-phenylindole (DAPI) or Hoechst for 5 min. Images were taken using a Leica fluorescent microscope and number of follicles with foci for pATM and γH2AX were analyzed using ImageJ software (NCBI). 5 large primary and 3 secondary follicles were quantified per slide (n=3 ovaries; 15 large primary and 9 secondary follicles quantified per ovary).

**Statistical analysis**

Raw data were analyzed by paired t-tests comparing treatment with control using Graphpad Prism 5.04 software. Values are expressed as mean ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05.

**Results**

**Effect of DMBA on Caspase-3 protein level**

F344 female rat PND4 pup ovaries were cultured in medium containing vehicle control (DMSO) for four days, followed by a single exposure to vehicle control medium ± DMBA
(12.5 nM or 75 nM) and maintained in culture for a further 4 or 8 days. The effect of DMBA on the protein level of an apoptosis marker, caspase-3, was determined. Caspase-3 protein level (17 Kda) was increased \((P < 0.05)\) by both DMBA treatments (CT - 1.0 ± 0.02; 12.5 nM - 1.12 ± 0.03; 75 nM - 1.15 ± 0.03), after 8 days confirming that apoptosis was induced by DMBA exposure (Figure 1).

**Localization and quantification of DMBA-induced markers of DNA damage**

F344 female rat PND4 pup ovaries were cultured in medium containing vehicle control (DMSO) for four days, followed by a single exposure to vehicle control medium +/- DMBA (12.5 nM or 75 nM) and maintained in culture for a further 4 or 8 days. Immunofluorescence staining was used to determine localization and staining intensity for pATM and γH2AX proteins. γH2AX protein was localized in granulosa cells of CT ovaries at both time points, but increased in the oocyte nucleus of large primary and secondary follicles following DMBA treatment (Figure 2A-C). Furthermore, Western blotting demonstrated that γH2AX protein level was also increased \((P < 0.05)\) after 2 days of 1 µM DMBA exposure (Figure 2D).

**Ovarian DMBA exposure alters expression of genes involved in DNA repair**

To determine the effect of DMBA on DNA repair gene mRNA expression, F344 female rat PND4 pup ovaries were cultured in medium containing vehicle control (DMSO) for 4 days, followed by a single exposure to vehicle control medium +/- DMBA (12.5 nM or 75 nM) and maintained in culture for a further 4 or 8 days. After exposure to DMBA, RNA was isolated and qRT-PCR was performed to determine mRNA expression of DNA repair genes. Relative to control treated ovaries, \(Atm\) (0.9-fold ± 0.1), \(Xrcc6\) (1.6-fold ± 0.3), \(Brca1\) (1.4-fold ± 0.4) and \(Rad51\) (0.6-fold ± 0.1) mRNA were increased \((P < 0.05)\) by the 12.5 nM
DMBA treatment after 4 days of exposure. In contrast, *Rad51* (0.7-fold ± 0.2) mRNA was decreased (*P* < 0.05) only by treatment with 75 nM DMBA. *Parp1* mRNA expression was decreased (*P* < 0.05) by both 12.5 (0.75-fold ± 0.04) and 75 nM (0.89-fold ± 0.06) DMBA concentrations after 4 days (Figure 3A). After 8 days of both DMBA treatments, mRNA encoding *Atm* (12.5 nM: 0.84-fold ± 0.2; 75 nM: 0.3-fold ± 0.03) and *Parp1* (12.5 nM: 0.3-fold ± 0.1; 75 nM: 0.7-fold ± 0.3) were increased (*P* < 0.05) compared to control treated ovaries (Figure 3B).

To further confirm that the DNA repair response is activated prior to follicle depletion, F344 female rat PND4 pup ovaries were cultured in medium containing vehicle control (DMSO) or DMBA (1μM) for 2 days. At this concentration, DMBA induces primordial and small primary follicle loss from 4 days of exposure onwards (Igawa *et al.*, 2009). Relative to control treated ovaries, *Atm* (1.18-fold ± 0.4), *Parp1* (0.78-fold ± 0.1), *Brca1* (0.55-fold ± 0.2) and *Rad51* (3.07-fold ± 0.7) mRNA were increased (*P* < 0.05) by 1 μM DMBA (Figure 3C).

**Ovarian DMBA exposure alters DNA repair protein level**

F344 female rat PND4 ovaries were cultured in medium containing vehicle control (DMSO) for 4 days, followed by a single exposure to vehicle control medium ± DMBA (12.5 nM or 75 nM) and maintained in culture for a further 4 or 8 days at which point total protein was isolated. Total ATM protein was measured by Western blotting and was increased (*P* < 0.05) after 4 days in ovaries treated with 75 nM DMBA (CT - 0.93 ± 0.03; 12.5 nM - 0.96 ± 0.03; 75 nM - 1.17 ± 0.04; Figure 4A), and after 8 days in ovaries exposed to 12.5 nM DMBA (CT - 0.53 ± 0.01; 12.5 nM - 0.66 ± 0.01; 75 nM - 0.57 ± 0.03; Figure 4A).
pATM protein was observed in the nucleus and cytoplasm of oocytes and in granulosa cells of all type of follicles (Figure 5A-C). There was no effect of 12.5 nM DMBA on pATM protein staining, relative to control. However, pATM was increased \((P < 0.05)\) by 75 nM DMBA in large primary \((CT - 0.333 ± 0.33; 75 \text{ nM} - 1.67 ± 0.33)\) and secondary follicles \((CT - 2 ± 1.5; 75 \text{ nM} - 9.33 ± 1.3)\) (Figure 5D).

Western blotting demonstrated that PARP1 protein level increased \((P < 0.05)\) after 4 days when exposed to 12.5 nM DMBA \((CT - 1.14 ± 0.01; 12.5 \text{ nM} - 1.44 ± 0.07; 75 \text{ nM} - 1.10 ± 0.002; \text{ Figure 6A})\), and was increased \((P < 0.05)\) by both DMBA exposures after 8 days \((CT - 0.94 ± 0.02; 12.5 \text{ nM} - 1.03 ± 0.03; 75 \text{ nM} - 1.01 ± 0.01; \text{ Figure 6A})\).

**Discussion**

DMBA causes depletion of follicles at all stages of development (Rajapaksa et al., 2007; Igawa et al., 2009). Our previous work established that a single exposure to 12.5 or 75 nM DMBA caused loss of large primary and/or secondary follicles (Madden et al., under review), and thereby established a model to study pre-ovulatory follicle loss. In the current study, we first confirmed that DMBA induced apoptosis at these concentrations by evaluating the level of caspase-3 in the ovary, which increased during DMBA exposure. Having established that atresia is ongoing in the ovary 8 days after DMBA exposure, we determined the impact of DMBA on DNA repair proteins as a proxy for DNA damage.

DMBA directly or indirectly causes DNA damage in extra-ovarian tissues (Rajasekaran et al., 2011), which can induce different cell fates such as DNA repair, cell cycle arrest and apoptosis (Norbury and Hickson, 2001). While DMBA-induced DNA damage has been characterized in many cell types (Bolognesi et al., 1991; Muqbil et al.,
2006; Rajasekaran et al., 2011), the ovarian mechanisms are not well understood. DNA DSB is highly cytotoxic lesions, generated by DMBA (Muqbil et al., 2006) and DSB’s are one of the mechanisms that cause phosphoramide mustard-induced follicle loss in ovaries (Petrillo et al., 2011). We localized γH2AX protein to the nucleus and granulosa cells of large primary and secondary follicles after both DMBA exposures; however this staining was negligible in the oocyte nucleus of control-treated ovaries. Additionally, we demonstrated that γH2AX protein is increased in ovaries exposed for two days to a concentration of DMBA that causes primordial and small primary depletion after 4 days. Since, detection of γH2AX is considered the gold standard for localization of DNA DSBs (Svetlova et al., 2010; Petrillo et al., 2011) these data indicate that DNA damage is occurring in ovaries exposed to DMBA.

The PI3K family member ATM is described as a critical sensor to initiate the cellular genotoxic response after the occurrence of DSBs (Shiloh, 2003; Yang et al., 2003; Gao et al., 2008). In this study, we determined an increase in the level of mRNA encoding Atm by exposure to both DMBA treatments. Total ATM protein was also increased by DMBA exposure, thus potentially also serving as a sensor of DNA DSB occurrence in the ovary. Interestingly, there was a temporal pattern of ATM protein induction, with the 75 nM DMBA concentration demonstrating induction of ATM after 4 days, while the 12.5 nM DMBA exposure took a longer time for ATM induction to occur. Whether the 12.5 nM exposure caused DNA damage prior to the induction of ATM cannot be confirmed from the data herein but is possible. These data are consistent with a previous study that demonstrated increased ATM in WT female mice spleen cells exposed to 50 mg/kg DMBA (Gao et al., 2008). ATM has been localized to the cytoplasm of growing follicles in the mouse ovary (Barlow et al., 1998). We also observed localization of pATM to the oocyte cytoplasm and
nucleus of large primary and secondary follicles further supporting that DMBA induces DNA damage in the rat ovary. The ATM pathway regulates cell survival by either inducing apoptosis or preventing cell progression and activating DNA repair (Lavin and Kozlov, 2007). ATM can initiate the action of the HR or the NHEJ DNA repair machinery components in the mice embryo (Chiruvella et al., 2012) and in MCF7 cells after exposure to ionizing radiation (Paull et al., 2000; Summers et al., 2011), respectively. In this study, Xrcc6, Brca1 and Rad51 mRNA were increased by exposure to the concentration of DMBA at which both large primary and secondary follicles are depleted, and Brca1 and Rad51 mRNA were increased by 1 µM DMBA exposure after 2 days. Since these are components of the HR pathway that predominates in actively dividing cells, these data could suggest that the DMBA-induced DNA repair response occurs in granulosa cells. However, it has also been recently shown that genes involved in HR are expressed in the oocyte of mice and humans, and that their expression levels decline as the female ages (Titus et al., 2013).

In response to DNA damage, cells can also activate PARP1, an enzyme that catalyzes poly-ADP-ribosylation of a variety of proteins (D'Amours et al., 1999). Activation of PARP1 can also increase the accessibility of DNA repair enzymes and transcription factors to chromatin (Dantzer et al., 2006). In this study, Parp1 mRNA and protein were increased by both concentrations of DMBA. Also, Parp1 mRNA was observed to be increased prior to follicle loss in the ovaries exposed to 1 µM DMBA. There has been controversy concerning the role of PARP1 in the regulation of cell survival/death in response to DNA damage. Some studies have implicated PARP1 in the regulation of DNA repair and cell survival (Wang et al., 1997; Ziegler and Oei, 2001; De Vos et al., 2012) whereas others have implicated PARP1 in initiating cell death by either apoptosis (Yu et al., 2002; Muñoz-Gámez et al.,
2009) or necrosis (Ha and Snyder, 1999). The data reported herein may indicate PARP1 activation may stimulate cell death after DMBA exposure.

In summary, this study indicates that exposure to DMBA causes ovotoxicity through DSB formation, and that the ovary mounts a protective response in order to repair this damage. The response to DSB’s however is not sufficient to completely prevent pre-ovulatory follicle loss at the DMBA levels investigated however it is possible that this is the case at lower exposure levels. Taken together these data raise concern about the fertility impacts of low level DMBA exposure to females.

Conflict of Interest Statement

The project described was supported by award number R00ES016818 to AFK and by a fellowship from the American Association of University Women to SG. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.

References


**Table 1. Primer sequence used for qRT-PCR**

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<th>Genes</th>
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<th>Reverse primer</th>
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<td>AGACAGACATGCTGCCTCCT</td>
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<tr>
<td><em>Xrcc6</em></td>
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Figure 1. Effect of DMBA exposure on caspase-3 protein expression

Following 8 d of culture, total protein was isolated from PND4 rat ovaries exposed to control (CT), 12.5 or 75 nM DMBA. Caspase-3 protein was measured by Western blotting. (A) Densitometry data was normalized to Ponceau S and expressed as mean raw data ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05. (B) Western blotting of caspase-3. DMBA increases Caspase 3 protein level during follicle loss.
Figure 2. Localization and quantification of γH2AX protein

Paraffin embedded ovarian sections from PND4 rat ovaries exposed to (A) control (CT), (B) 12.5 or (C) 75 nM DMBA were used to perform immunohistochemistry to determine localization of γH2AX protein after 4 d. (D) Quantification of γH2AX loci in large primary and secondary follicles; Data is expressed as number of follicles positive for γH2AX ± SE; n=3; Statistical significance was defined as * = P < 0.05. (E) Western blot to detect γH2AX in vehicle control (C) or 1 µM DMBA (D) treated ovaries and expressed as mean raw data ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05. γH2AX staining increases at 75 nM DMBA at 4 days.
Figure 3. Effect of DMBA exposure on DNA repair gene mRNA expression

Following (A) 4 or (B) 8d of culture, RNA was isolated from PND4 rat ovaries exposed to control (CT), 12.5 or 75 nM DMBA and qRT-PCR performed. Additionally, RNA was isolated from ovaries exposed to (C) control (CT) or 1 µM DMBA for qRT-PCR. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05. DMBA alters DNA repair gene expressions.
Figure 4. Effect of DMBA exposure on total ATM protein level

Following 4 or 8d of culture, total protein was isolated from PND4 rat ovaries exposed to control (CT), 12.5 or 75 nM DMBA. ATM protein was measured by Western blotting. (A) Results were normalized to Ponceau S and expressed as mean raw data ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05. (B) Representative western blot for ATM. DMBA alters ATM protein level.
Figure 5. Localization and quantification of pATM protein

Following 8d of culture, paraffin embedded ovarian sections from PND4 rat ovaries exposed to (A) control (CT), (B) 12.5 or (C) 75 nM DMBA were used to perform immunohistochemistry to determine localization and (D) quantification of pATM protein. Data is expressed as number of follicles positive for pATM ± SE; n=3; Statistical significance was defined as * = P < 0.05. pATM staining increases at higher concentration in pre-antral follicles.
Figure 6. Effect of DMBA exposure on PARP1 protein level

Following 4 or 8d of culture, total protein was isolated from PND4 rat ovaries exposed to control (CT), 12.5 or 75 nM DMBA. PARP1 protein was measured by Western blotting. (A) Results were normalized to Ponceau S and expressed as mean raw data ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05. (B) Representative Western blot for PARP1. PARP1 protein increases by DMBA exposure.
CHAPTER 3. 7,12-DIMETHYLBENZ[A]ANTHRACENE INDUCES THE DNA DAMAGE RESPONSE IN OVARIES OF EXPOSED MICE, WITH ADDITIVE IMPACT OF OBESITY

A paper submitted to Toxicology and Applied Pharmacology (Accepted)

Ganesan, S., Nteeba, J., Keating, A.F.

Contribution Statement:
I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Nteeba, J. aided with designing and performing the experiment and tissue collection. Keating, A.F. designed the experiments, aided in data interpretation and edited the manuscript.

Abstract

7,12-dimethylbenz[a]anthracene (DMBA) reduces ovarian follicle number, and induces DNA damage in extra-ovarian tissues, thus, this study investigated ovarian DMBA-induced DNA damage. Additionally, since obesity is associated with increased incidence of offspring birth defects, we hypothesized that a DMBA-induced DNA damage response (DDR) might be compromised in ovarian tissue from obese females. Wild type (lean) non agouti (a/a) and KK.Cg-Ay/J heterozygote (obese) mice were dosed with sesame oil or DMBA (1mg/kg; intraperitoneal injection) at 18 wks of age, for 14 days. Total ovarian RNA and protein were isolated and abundance of Ataxia telangiectasia mutated (Atm), X-ray repair complementing defective repair in chinese hamster cells 6 (Xrcc6), Breast cancer type 1 (Brca1), Rad 51 homolog (Rad51), Poly [ADP-ribose] polymerase 1 (Parp1) and Protein kinase, DNA-activated, catalytic polypeptide (Prkdc) quantified by RT-PCR or Western blot, respectively. Phosphorylated histone H2AX (γH2AX) level was also determined by Western blotting. Basal protein abundance of PRKDC and BRCA1 proteins were decreased ($P < 0.05$) but γH2AX and PARP1 proteins increased ($P < 0.05$) in ovaries from obese females. Ovarian
ATM, XRCC6, PRKDC, RAD51 and PARP1 proteins were increased \((P < 0.05)\) by DMBA exposure in lean mice. A blunted DMBA-induced increase \((P < 0.05)\) in XRCC6, PRKDC, RAD51 and BRCA1 was observed in ovaries from obese mice, relative to lean counterparts. Taken together, DMBA exposure induced \(\gamma\text{H2AX}\) as well as the ovarian DDR, supporting that DMBA causes ovarian DNA damage as an ovotoxic insult. Additionally, the ovarian DDR was partially attenuated in obese females raising concern that obesity may be an additive factor during chemical-induced ovotoxicity.

**Introduction**

7,12-dimethylbenz[a]anthracene (DMBA) is a polycyclic aromatic hydrocarbon liberated through cigarette smoke and car exhaust fumes (Gelboin, 1980). DMBA destroys all follicle types leading to ovarian failure in mice and rats (Mattison and Schulman, 1980). In addition, it is recognized that female smokers experience ovarian senescence at an earlier age than their non-smoking counterparts (Jick and Porter, 1977). DMBA requires the action of ovarian enzymes cytochromes p450 (Cyp) isoforms 1A1 and 1B1 (Shimada et al., 2003) and microsomal epoxide hydrolase (mEH) (Rajapaksa et al., 2007; Igawa et al., 2009; Madden et al., 2014) for biotransformation to the ovotoxic metabolite, DMBA 3,4-diol, 1,2-epoxide, which is both carcinogenic and has the potential to form DNA adducts (Miyata et al., 1999). DMBA exposure also induces the DNA damage response (DDR) in cultured neonatal rat ovaries, indicating that DNA damage is a potential mechanism by which DMBA induces its ovotoxic effects (Ganesan et al., 2013).

Double-strand breaks (DSBs) in DNA are cytotoxic lesions, generated by ionizing radiation and man-made chemicals (van Gent et al., 2001). DSB’s can be sensed by a PI3K
family member Ataxia telangiectasia mutated (ATM) protein (Norbury and Hickson, 2001; Yang et al., 2003; Giunta et al., 2010). ATM phosphorylates histone H2AX (γH2AX) which leads to recruitment of DNA repair molecules to the site of DSBs (Svetlova et al., 2010), thus γH2AX become a gold standard marker for localizing DSBs. DNA DSB’s pose a serious threat to both cell viability and genome stability if left unrepaired or repaired incorrectly, and could potentially lead to permanent damage with resulting negative consequences for gamete health (Petrillo et al., 2011; Summers et al., 2011). Two major pathways can repair DNA DSB’s: Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). Key signaling molecules involved in NHEJ repair include the X-ray repair complementing defective repair in Chinese hamster cells 6 and 5 (XRCC6 and XRCC5) heterodimer which recognizes and binds to the DSB, recruiting protein kinase DNA-activated, catalytic polypeptide (PRKDC) to the DSB ends. During HR, breast cancer type 1 (BRCA1) can be phosphorylated by ATM and co-localizes with RAD51 recombinase (RAD51) at the site of DNA damage to induce DSB repair (Petrillo et al., 2011).

Approximately 65% of women in the United States are overweight or obese (Flegal Km et al., 2010) and the associated reproductive complications include menstrual cycle disturbances, ovulatory dysfunction, infertility, decreased conception, early pregnancy loss and congenital abnormalities in offspring (Cardozo et al., 2012; Sauber-Schatz et al., 2012). Obesity has been associated with altered insulin and insulin growth factor (IGF) signaling (Qatanani and Lazar, 2007). Insulin serves as a regulator of enzymes involved in the metabolism of xenobiotics (Woodcroft and Novak, 1999), mediated, at least partly, through activation of phosphatidylinositol-3 kinase (PI3K) and variety of downstream effectors including protein kinase B (Akt) (Niswender et al., 2003; Kim and Novak, 2007). Insulin
increases mEH protein expression in primary cultured rat hepatocytes (Kim et al., 2003). Also, increased ovarian PI3K signaling and mEH have been demonstrated in mice fed a high fat diet until obese (Nteeba et al., 2013). The lethal yellow mouse, which develops progressive obesity, was found to have both higher basal levels of mEH and greater mEH induction in response to DMBA exposure (Nteeba et al., 2014). These data suggest that ovarian tissue from obese females could have potentially greater exposure to the ovotoxic metabolite of DMBA, due to higher levels of ovarian mEH, and thus greater DMBA bioactivation to a metabolite that interacts with DNA (Miyata et al., 1999). The objective of this study was therefore to investigate ovarian DMBA-induced DNA damage as evidenced by phosphorylation of H2AX and induction of the DDR in both lean and obese mice.

Methods and Materials

Reagents

7,12-dimethylbenz[a]anthracene (DMBA; CAS # 57-97-6), sesame oil (CAS # 8008-74-0), 2-ß-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N’N’N’-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, Sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA) with the exception of the BRCA1(C-20) primary antibody which was from Santa Cruz Biotechnology (Santa Cruz, CA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz
Biotechnology (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

Animals

Ovarian tissue was obtained as part of a larger study by our group (Nteeba et al., 2014). Briefly, four week old female wild type normal non-agouti (a/a; designated lean; n = 10) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 10) were purchased from Jackson laboratories (Bar Harbor, ME 002468). All animals were housed in cages under a 12 h light/dark photoperiod with the temperature between 70-73°F and humidity approximately 20-30%. The animals were provided with a standard diet (Teklad 2014 global 14% protein rodent maintenance diet) with ad libitum access to food and water until 18 weeks of age. All animal experimental procedures were approved by the Iowa State University Animal Care and Use Committee.

In vivo DMBA exposure

Both lean and obese mice were intraperitoneally (i.p) dosed with sesame oil (SO) or DMBA (95%; 1mg/kg) for 14 days. This dose was chosen based on the literature (Mattison and Thorgeirsson, 1979). Mice were euthanized 3 days after the end of dosing in their proestrus phase. One ovary from each mouse was fixed in 4% paraformaldehyde and one ovary was preserved in RNA later at -80°C for RNA and protein isolation. No difference in body weight due to DMBA exposure was observed and the lethal yellow mice had higher body weights (Nteeba et al., 2014). DMBA reduced ovarian weight and volume in both lean and obese mice, relative to vehicle treated mice, and ovarian weight and volume was lower in obese DMBA-treated relative to lean DMBA-treated mice (Nteeba et al., 2014).
RNA isolation and qRT-PCR

RNA was isolated using an RNeasy Mini kit (Qiagen) and the concentration was determined using an ND-1000 Spectrophotometer (\(\lambda = 260/280\)nm; NanoDrop technologies, Inc., Wilmington, DE) (n=3). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). cDNA was diluted (1:20) in RNase-free water. Diluted cDNA (2 \(\mu\)l) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers for Atm, Brca1, Prkdc, Parp1, Rad51, Xrcc6 and Gapdh were designed by Primer 3 Input Version (0.4.0) and are listed in Table 1. The regular cycling program consisted of a 15-min hold at 95\(^\circ\)C and 45 cycles of denaturing at 95\(^\circ\)C for 15s, annealing at 58\(^\circ\)C for 15s, and extension at 72\(^\circ\)C for 20s at which point data were acquired. There was no difference in Gapdh mRNA expression between treatments, thus each sample was normalized to Gapdh before quantification. Quantification of fold-change in gene expression was performed using the 2\(^{-\Delta\Delta Ct}\) method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Protein isolation and western blotting

Protein was isolated from whole ovaries (n=3) by homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Thompson et al., 2005). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a standard BCA protocol. SDS-PAGE was used to separate protein homogenates which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 5% milk in Tris-buffered saline containing tween 20, followed by incubation with one of: anti-rabbit PARP1 antibody (1:200), anti-rabbit phosphorylated H2AX antibody (\(\gamma\)H2AX; 1:100),
anti-mouse ATM antibody (1:100), anti-mouse RAD51 antibody (1:500), anti-mouse XRCC6 antibody (1:100), anti-rabbit BRCA1 antibody (1:500), or anti-rabbit PRKDC antibody (1:100) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with species-specific secondary antibodies (1:2000) for 1h at room temperature. Membranes were washed 3X in TTBS and incubated in chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Chemical exposures can impact traditional housekeeping protein abundance (our unpublished data), thus equal protein loading was confirmed by Ponceau S staining of total protein and protein level was normalized to Ponceau S densitometry values.

**Statistical analysis**

Raw data were analyzed by unpaired t-test (comparison of two samples) or one-way ANOVA (comparison of multiple samples) analysis with the Tukey pairwise comparison using Graphpad Prism 5.04 software. Values are expressed as mean ± SE; n=3. A P-value < 0.05 was considered a significant difference between treatments, while P < 0.1 was considered a trend towards a difference.

**Results**

**Effect of DMBA on ovarian H2AX phosphorylation in lean and obese mice**

Phosphorylation of H2AX occurs within seconds of a DNA DSB and is considered the gold standard marker for DSB detection. γH2AX protein was absent in lean control-treated ovaries but was evident (P < 0.05) in obese ovaries that had received sesame oil. γH2AX
protein level was increased \((P < 0.05)\) by DMBA exposure in both lean and obese mice compared to their respective control-treated ovaries (Figure 1A). Relative to lean mice, the DMBA-induced increase in \(\gamma H2AX\) protein was lower in ovaries from obese mice (Figure 1B).

**Impact of DMBA exposure on ovarian Atm abundance in lean and obese mice**

ATM is the cellular sensor of DNA DSBs. Basal \(Atm\) mRNA levels were lower \((P < 0.05)\) in ovaries from obese relative to lean mice. In lean mice, ovarian \(Atm\) mRNA levels were decreased \((P < 0.05)\) by DMBA exposure compared to control-treated animals. In contrast, in obese mice, DMBA exposure increased \((P < 0.05)\) ovarian \(Atm\) mRNA levels relative to control-treated obese mice (Figure 2A). There was no difference in basal ovarian ATM protein levels between lean and obese mice. Additionally, DMBA increased \((P < 0.05)\) ATM protein levels in both lean and obese mice to the same extent (Figure 2B, C).

**DMBA-induction of ovarian Xrcc6 mRNA and protein in lean and obese mice**

\(Xrcc6\) is a member of the NHEJ pathways recruited by \(\gamma H2AX\). Basal levels of \(Xrcc6\) mRNA were lower \((P < 0.05)\) in obese compared to lean mouse ovaries. In lean mice, levels of \(Xrcc6\) mRNA were increased by DMBA exposure while, in contrast, DMBA exposure did not impact \(Xrcc6\) in obese ovaries (Figure 3A). There was no impact of obesity on ovarian XRCC6 protein. In lean and obese mice, relative to control-treated animals, levels of ovarian XRCC6 protein were increased \((P < 0.05)\) by DMBA exposure. Interestingly, relative to lean DMBA-treated ovaries, DMBA-induced XRCC6 protein expression was lower \((P < 0.05)\) in obese ovaries (Figure 3B, C).
**Response of ovarian Prkdc mRNA and protein to DMBA exposure in lean and obese mice**

The NHEJ member, PRKDC, is recruited by γH2AX [20] upon DSB formation. Basal levels of Prkdc mRNA were lower \((P < 0.05)\) in obese compared to lean ovaries. In lean mice, levels of Prkdc mRNA were decreased \((P < 0.05)\) by DMBA exposure compared to control-treated ovaries. In contrast, DMBA exposure increased \((P < 0.05)\) Prkdc mRNA level in ovaries from obese mice, compared to obese control-treated animals (Figure 4A). Basal PRKDC protein levels were also lowers \((P < 0.05)\) in obese relative to lean ovaries. In both lean and obese mice, relative to control-treated animals, levels of ovarian PRKDC protein were increased \((P < 0.05)\) by DMBA exposure (Figure 4B, C).

**Impact of DMBA exposure on ovarian Brca1 mRNA and protein in lean and obese mice**

BRCA1 is phosphorylated by ATM during HR. Lower \((P < 0.05)\) levels of basal Brca1 mRNA were observed in obese ovaries relative to lean ovaries. In both lean and obese mice, levels of Brca1 mRNA were increased \((P < 0.05)\) by DMBA exposure compared to control treatments, however, obesity resulted in a blunted DMBA-induced Brca1 mRNA increase (Figure 5A). Basal BRCA1 protein levels were lower \((P < 0.05)\) in obese relative to lean ovaries. In obese mice, relative to control-treated animals, levels of ovarian BRCA1 protein were increased \((P < 0.05)\) by DMBA exposure while no BRCA1 induction was observed in lean mice (Figure 5B, C).

**Ovarian DMBA-induced Rad51 mRNA and protein in lean and obese mice**

RAD51 is also part of HR and activated upon DSB formation. There was a trend \((P = 0.07)\) for reduced Rad51 mRNA in obese relative to lean ovaries. In lean and obese mice, levels of Rad51 mRNA were increased by DMBA exposure compared to control treatments, however, the DMBA-induced increased in Rad51 mRNA level was reduced \((P < 0.05)\) in
ovaries, relative to the response in lean ovaries (Figure 6A). There was no difference in basal RAD51 protein levels between lean and obese ovaries. In lean and obese mice, RAD51 protein levels were increased ($P < 0.05$) by DMBA exposure compared to control treatments, however, the DMBA-induced RAD51 protein level was lower ($P < 0.05$) in obese relative to lean ovaries (Figure 6B, C).

**DMBA-induction of ovarian Parp1 mRNA and protein in lean and obese mice**

PARP1 is another protein involved in the response to DSB. Higher basal levels of Parp1 mRNA were noted in obese compared to lean ovaries. In lean and obese mice, levels of Parp1 mRNA were increased ($P < 0.05$) by DMBA exposure compared to control treatments (Figure 7A). Basal PARP1 protein levels were higher in obese relative to lean ovaries. DMBA increased ($P < 0.05$) PARP1 protein in both lean and obese mice (Figure 7B, C).

**Discussion**

Increased bioactivation of DMBA by elevated ovarian mEH during obesity (Nteeba et al., 2014) could induce DNA damage since DMBA activation is mediated through mEH, resulting in generation of an ovotoxic alkylating metabolite containing two epoxide groups (Miyata et al., 1999). Thus, we assessed the formation of DSBs as well as the DDR in the ovaries of DMBA-exposed mice. The dose and route of DMBA exposure was chosen based on a previous study (Mattison and Thorgeirsson, 1979) which was shown to be effective in destroying approximately 50% of the primordial follicle pool. Female cigarette smokers are known to undergo ovarian failure earlier than their non-smoking counterparts (Jick and Porter, 1977), however, since it is difficult to extrapolate human ovarian exposure to DMBA
due to differences in individual hepatic xenobiotic biotransformation a physiological endpoint provides a bioassay approach for measurement of ovarian impacts. This physiological endpoint of approximately 50% primordial follicle depletion allows for the study of changes to the ovary in the absence of complete depletion of the follicular reserve.

Obesity is a detrimental physiological state for oocyte quality and fertility (Wittemer et al., 2000; Brewer and Balen, 2010). Maternal obesity increases the risk of offspring birth defects (Watkins et al., 2003). Interestingly, poor quality oocytes (Cardozo et al., 2012) and birth defects are also caused by DNA damage in germ cells (Flegal KM et al., 2010; Sauber-Schatz et al., 2012). The obese mice had elevated basal glucose and had a compromised response to a glucose tolerance test (data not shown; (Nteeba et al., 2014). DMBA depleted healthy follicles of all types in both lean and obese treated females compared to sesame-treated females, and ovarian weight was lower in the obese DMBA relative to the lean DMBA treated mice, indicating greater levels of ovotoxicity (Nteeba et al., 2014). Since DMBA potentially causes ovarian DNA damage and because we observed lower ovarian weights in DMBA exposed ovaries from obese mice, relative to their lean counterparts (Nteeba et al., 2014), we determined whether obesity has any additive impact on DMBA-induced ovotoxicity.

ATM is recruited upon DSB formation to phosphorylate downstream cellular effector molecules resulting in either cell cycle arrest to accommodate DNA repair (Canman et al., 1998; Lee and Paull, 2007) or apoptosis (Stankovic et al., 2002) (Waster and Ollinger, 2009). DMBA exposure decreased Atm mRNA level in lean ovaries while the opposite was the case in obese ovaries. Despite this, increased ATM protein was observed in both lean and obese ovaries upon DMBA exposure indicating that ovarian DNA damage is imparted by DMBA
exposure. Activation of ATM, associated with apoptotic oocyte death, has been previously observed during doxorubicin exposure in embryonic stem cells (Soleimani et al., 2011). While it is difficult to conclude whether our conflicting effects of DMBA exposure in lean and obese ovaries on Atm mRNA levels indicate different mechanisms of regulation during obesity, it is clear that DMBA exposure increased ovarian ATM protein regardless of metabolic status, supporting that DMBA induces ovarian DNA damage.

When a DSB occurs, ATM phosphorylates H2AX (γH2AX), a sensitive marker of DSBs (Bouquet et al., 2006; Mah et al., 2010). We demonstrate increased γH2AX protein due to DMBA exposure regardless of the presence of obesity. This data are in agreement with our previous study that found activated γH2AX protein in neonatal rat ovaries that had been exposure to DMBA (Ganesan et al., 2013). Interestingly, low levels of γH2AX protein were observed during obesity, independent of DMBA exposure, which may indicate that low level DNA damage is present in ovarian tissue of obese females. DNA damage in peripheral lymphocytes of obese and overweight italian children has been demonstrated (Jungheim et al., 2010), thus our data is in agreement with these studies. Taken together, this data further supports that DMBA exposure induces ovarian DNA damage, and that the ovary is responsive to such an insult.

γH2AX recruits XRCC6 and PRKDC to repair DNA by the NHEJ pathway (Summers et al., 2011). Xrcc6 mRNA was increased by DMBA exposure in lean ovaries but no change was observed with obesity, though a lower basal level was present. In contrast to mRNA data, XRCC6 protein was increased by DMBA in both lean and obese ovaries but this increase appeared to be blunted due to obesity. This lowered response could be due to basal DNA damage that is seemingly present in ovarian tissue during obesity as suggested by the
observed γH2AX. Defective DNA repair and chromosomal instability due to decreased XRCC6 have been reported in embryonic cells (Gu et al., 1997). In addition, suppressed XRCC6 leads to DNA damage and reactive oxygen species-induced endoplasmic reticulum stress in Toll like receptor (TLR) 4 mutant liver cells, ultimately leading to induction of the ATM-PRKDC protein complex as well as induction of PARP1, resulting in apoptosis of precancerous hepatocytes (Wang et al., 2013). Prkdc mRNA expression was decreased by DMBA exposure in lean but increased in obese ovaries. In contrast, despite their being reduced basal levels of PRKDC protein in obese relative to lean mice, PRKDC protein induction by DMBA exposure was observed in both lean and obese mice. Inhibition of PRKDC reduces H2AX phosphorylation and the HR repair mechanism in a medaka embryonic cell line (Urushihara et al., 2012). Thus, our data suggest that increased PRKDC by DMBA exposure might play a pro-apoptotic role in ovarian tissue.

PARP1 is involved in DNA damage-induced cell apoptosis (D'Amours et al., 1999) and DMBA has been shown to increase PARP1 expression prior to follicle loss in neonatal rat ovaries (Ganesan et al., 2013). DMBA increased both Parp1 mRNA and protein in lean and obese ovaries, with ovaries from obese mice having higher basal levels of Parp1. Elevated Parp1 could play a role in DMBA-induced ovotoxicity, since PARP1 inhibition previously protected against cisplatin-induced renal damage and inflammation (Kim et al., 2012). Interestingly, the mitochondrial apoptotic pathway mediated by caspase 9 is constitutively activated in oocytes and contributes to the elimination of oocytes with meiotic errors through the cleavage and activation of PARP1 (Ene et al., 2013). Thus, increased PARP1 observed in our study could mediate increased DMBA-induced ovotoxicity.
RAD51 interacts with BRCA1 during DDR and DMBA exposure increased RAD51 protein abundance in both prostate tissue (Xu et al., 2002) and in cultured neonatal ovaries from rats (Ganesan et al., 2013). In this study, DMBA exposure increased Rad51 mRNA levels in lean but not obese mice. Furthermore, ovarian RAD51 protein was upregulated by DMBA in the lean females but a reduced response was observed in ovaries from obese females. DMBA has been shown to induce ROS generation and DNA damage in Brca1 knock down MCF10A cells (Kang et al., 2013). In agreement with our previous study demonstrating increased Brca1 in neonatal rat ovaries exposure to DMBA (Ganesan et al., 2013), we found increased DMBA-induced Brca1 mRNA in ovaries of lean mice but this response was partly attenuated in ovaries from obese mice. In contrast, BRCA1 protein was increased in ovaries from obese but not lean ovaries due to DMBA exposure. Interestingly, and in contrast to increased γH2AX, we noted that basal Brca1 mRNA and protein abundance was lower due to obesity. Reduced Brca1 is associated with an increased risk of hereditary breast and ovarian cancer (Robson et al., 1998; Kauff et al., 2002). BRCA1 controls the mitotic checkpoint complex, and loss of this control can lead to mitotic errors contributing to neoplasia in ovarian cystadenoma cells (Yu et al., 2012). Further, women with Brca1 mutations can experience earlier ovarian senescence and develop ovarian cancer at younger ages (Rzepka-Górska et al., 2006). Impairment of BRCA1-related DNA DSB repair has been associated with accelerated loss of the ovarian follicular reserve and with accumulation of DSB in human oocytes, suggesting that DNA DSB repair efficiency is an important determinant of oocyte aging in women (Titus et al., 2013). Thus, any impact of DMBA on Brca1 levels could indicate potential negative female fertility consequences.
In summary, our results support that DMBA exposure induces DSB formation in the ovary and the DDR is activated in ovarian tissue of exposed mice. Interestingly, we found a disconnect between mRNA and protein levels, perhaps indicating that a delay in transcript processing occurs in ovaries from obese mice, or that other regulators of gene expression are affected potentially including microRNA as we have previously noted in a high fat feeding-induced obesity mouse model (Nteeba et al., 2013), underscoring the importance of investigating translational as well as transcriptional effects of obesity. It is important to note that this study represents a snapshot in time of the DMBA-induced ovarian DDR, however, it is concerning that the ovaries from obese female mice were observed to have a low level of γH2AX protein therein, indicating that DNA damage, outside of ovotoxicant exposure, may be present. Also, the ovarian DDR was blunted due to obesity, indicating a reduced ability to respond to stress-induced DNA damage. Taken together, the observations reported in this study could imply a higher sensitivity to and susceptibility for DMBA-induced ovotoxicity effects in obese females.

**Conflict of Interest Statement**

The project described was supported by award number R00ES016818 to AFK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
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### Table 1. Primer sequences used for qRT-PCR

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<thead>
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<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>AGACAGACATGCTGCCTCCTT</td>
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<tr>
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</tr>
<tr>
<td><em>Xrcc6</em></td>
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<td>TGCTTTCTCCTCCACTCCTT</td>
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Figure 1. Effect of DMBA on H2AX phosphorylation in lean and obese mice

Lean and obese mice were dosed with sesame oil (CT) or DMBA (1 mg/kg; i.p.) for 14 days, followed by (A) Western blotting to detect γH2AX. Protein abundance was quantified using ImageJ software. Values are expressed as raw data mean ± SE; n=3. Different letters indicate $P < 0.05$. Both DMBA and obesity increase DNA damage formation.
Lean and obese mice were dosed with sesame oil (CT) or DMBA (1 mg/kg; i.p.) for 14 days, followed by RNA or protein isolation. qRT-PCR or Western blotting were performed to quantify *Atm* (A) mRNA or (B,C) protein abundance. (A) *Atm* was normalized to *Gapdh* for quantification, and values are reported as fold-change (n = 3) relative to lean CT ovaries (set at 1). (B) Protein abundance was quantified using ImageJ software. Values are expressed as raw data mean ± SE; n=3. Different letters indicate $P < 0.05$. DMBA increases ATM protein levels.
Lean and obese mice were dosed with sesame oil (CT) or DMBA (1 mg/kg; i.p.) for 14 days, followed by RNA or protein isolation. qRT-PCR or Western blotting were performed to quantify *Xrcc6* (A) mRNA or (B,C) protein abundance. (A) *Xrcc6* was normalized to *Gapdh* for quantification, and values are reported as fold-change (n = 3) relative to lean CT ovaries (set at 1). (B) Protein abundance was quantified using ImageJ software. Values are expressed as raw data mean ± SE; n=3. Different letters indicate $P < 0.05$. DMBA increases XRCC6 protein levels.
Lean and obese mice were dosed with sesame oil (CT) or DMBA (1 mg/kg; i.p.) for 14 days, followed by RNA or protein isolation. qRT-PCR or Western blotting were performed to quantify *Prkd* (A) mRNA or (B,C) protein abundance. (A) *Prkd* was normalized to *Gapdh* for quantification, and values are reported as fold-change (n = 3) relative to lean CT ovaries (set at 1). (B) Protein abundance was quantified using ImageJ software. Values are expressed as raw data mean ± SE; n=3. Different letters indicate $P < 0.05$. Obesity blunted DMBA induced PRKDC protein levels.
Figure 5. Impact of DMBA exposure on ovarian Brca1 mRNA and protein in mice

Lean and obese mice were dosed with sesame oil (CT) or DMBA (1 mg/kg; i.p.) for 14 days, followed by RNA or protein isolation. qRT-PCR or Western blotting were performed to quantify Brca1 (A) mRNA or (B,C) protein abundance. (A) Brca1 was normalized to Gapdh for quantification, and values are reported as fold-change (n = 3) relative to lean CT ovaries (set at 1). (B) Protein abundance was quantified using ImageJ software. Values are expressed as raw data mean ± SE; n=3. Different letters indicate $P < 0.05$. Obesity decreases BRCA1 protein levels.
Figure 6. Ovarian DMBA-induced Rad51 mRNA and protein in mice

Lean and obese mice were dosed with sesame oil (CT) or DMBA (1 mg/kg; i.p.) for 14 days, followed by RNA or protein isolation. qRT-PCR or Western blotting were performed to quantify Rad51 (A) mRNA or (B,C) protein abundance. (A) Rad51 was normalized to Gapdh for quantification, and values are reported as fold-change (n = 3) relative to lean CT ovaries (set at 1). (B) Protein abundance was quantified using ImageJ software. Values are expressed as raw data mean ± SE; n=3. Different letters indicate $P < 0.05$. DMBA increases RAD 51 protein levels.
Figure 7. DMBA-induction of ovarian Parp1 mRNA and protein in mice

Lean and obese mice were dosed with sesame oil (CT) or DMBA (1 mg/kg; i.p.) for 14 days, followed by RNA or protein isolation. qRT-PCR or Western blotting were performed to quantify Parp1 (A) mRNA or (B,C) protein abundance. (A) Parp1 was normalized to Gapdh for quantification, and values are reported as fold-change (n = 3) relative to lean CT ovaries (set at 1). (B) Protein abundance was quantified using ImageJ software. Values are expressed as raw data mean ± SE; n=3. Different letters indicate P < 0.05. Both obesity and DMBA increase PARP1 protein levels.
CHAPTER 4. IMPACT OF 7,12-DIMETHYLBENZ[a]ANTHRACENE EXPOSURE ON CONNEXIN GAP JUNCTION PROTEINS IN CULTURED RAT OVARIIES


Shanthi Ganesan and Aileen F. Keating

Contribution Statement:
I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Aileen F. Keating designed the experiments, aided in data interpretation and edited the manuscript.

Abstract

7,12-dimethylbenz[a]anthracene (DMBA) destroys ovarian follicles in a concentration-dependent manner. The impact of DMBA on connexin (CX) proteins that mediate communication between follicular cell types was investigated. Postnatal day (PND) 4 Fisher 344 rat ovaries were cultured for 4 days in vehicle medium (1% DMSO) followed by a single exposure to vehicle control (1% DMSO) or DMBA (12.5 nM or 75 nM) and cultured for 4 or 8 days. RT-PCR or Western blotting was performed to quantify Cx37 or Cx43 mRNA or protein level, respectively. Immunofluorescence staining determined CX37 or CX43 level and localization. Cx37 mRNA and protein increased ($P < 0.05$) at 4 days of DMBA exposure. Relative to vehicle control-treated ovaries, mRNA encoding Cx43 decreased ($P < 0.05$) but CX43 protein increased ($P < 0.05$) at 4 days by both DMBA exposures. mRNA expression of pro-apoptotic p53 was decreased ($P < 0.05$) but no changes in Bax expression were observed after 4 days of DMBA exposures. In contrast, after 8 days, DMBA decreased Cx37 and Cx43 mRNA and protein but increased both $p53$ and Bax mRNA levels. CX43 protein was located between granulosa cells, while CX37 was located at the oocyte cell surface of all follicle
stages. These findings support that DMBA exposure impacts ovarian Cx37 and Cx43 mRNA and protein prior to and at the time of follicle loss. It is possible that such interference in follicular cell communication is detrimental to follicle viability, and may play a role in DMBA-induced follicular atresia.

**Introduction**

The female gamete, the oocyte, is encased in a follicular structure surrounded by granulosa cells and, as the follicle matures, also by theca cells. Primordial follicles are maintained in a dormant state until activation into the growing follicular pool (Hirshfield, 1991). In women, nearly 99% of ovarian follicles undergo degenerative changes by a process known as atresia (Hirshfield, 1991) and ovarian senescence (menopause) occurs when the finite pool of primordial follicles has become exhausted (Mattison and Nightingale, 1982; Broekmans et al., 2007; Hansen et al., 2008). Since primordial follicles cannot be regenerated (Hirshfield, 1991), chemical-induced depletion of this follicle pool can lead to infertility and premature ovarian failure (POF). A number of chemical classes can deplete follicles causing ovotoxicity, including the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA) (Mattison, 1980; Hoyer et al., 2009; Igawa et al., 2009).

DMBA causes DNA double strand breaks (DSBs) damage in oocytes and granulosa cells to cause follicle loss (Ganesan et al., 2013). DSBs activate ataxia-telangiectasia mutated (ATM) protein which phosphorylates P53, a tumor suppressor protein. P53 can then activate DNA repair proteins, induce cell cycle arrest or initiate apoptosis if the DNA damage proves to be irreparable. Phosphorylated P53 activates a number of downstream pro-apoptotic
molecules including Bax (Lane, 1992). BAX promotes apoptosis by binding to and antagonizing the action BCL-2 protein, resulting in release of cytochrome c and activation of caspases to induce apoptosis (Weng et al., 2005). We and others have previously demonstrated that ovaries exposed to DMBA have increased levels of caspase 3 protein (Tsai-Turton et al., 2007; Ganesan et al., 2013). Additionally, BAX-deficient mice ovaries are resistant to DMBA-induced primordial follicle destruction (Matikainen et al., 2001).

Granulosa:granulosa and granulosa:oocyte cell to cell communication are necessary for maintenance of follicular viability. Communication between these cells occurs through gap junction intra-cellular channels, which directly connect the cytoplasmic compartments of neighboring cells and allow exchange of ions, metabolites and second messenger such as Ca^{2+} and inositol phosphates (Goldberg et al., 2004). Gap junctions are involved in regulation of cellular growth, metabolism and differentiation (Sohl and Willecke, 2003; Wei et al., 2004) and ovarian folliculogenesis (Simon et al., 2006). The major ovarian gap junction proteins are connexin (CX) 37 and 43 (Kidder and Mhawi, 2002a). CX37 communicates from the oocyte to granulosa cell (Simon et al., 1997), while CX43 functions in intra-granulosa cell communication (Granot et al., 2002; Kidder and Mhawi, 2002a). CX37 is thought to be involved in follicular development and ovulation as well as luteal tissue growth, differentiation, and regression (Borowczyk et al., 2006). Cx37-null mouse oocytes suffer growth retardation and do not survive to become meiotically competent (Carabatsos et al., 2000). Follicle growth is also interrupted: Cx37^{−} granulosa cells form structures resembling corpora lutea in the absence of ovulation. CX43 levels are increased in granulosa cells following activation of follicular growth and maturation (Melton et al., 2001), while reduced granulosa cell expression of CX43 is linked to elevated apoptosis in porcine,
bovine (Johnson et al., 1999; Cheng et al., 2005) and avian (Krysko et al., 2004) species. Thus, CX37 and CX43 play important roles in the ovary to maintain follicular and oocyte viability and quality.

Little is known about the impact of ovotoxicant exposures on ovarian function, thus in this study we investigated any impact of DMBA on ovarian gap junction genes Cx37 and Cx43. We utilized a neonatal rat whole ovary culture method to determine the effect of a single DMBA exposure at two concentrations: 12.5 nM and 75 nM on Cx37 and Cx43 mRNA and protein level. These exposures have previously been shown to cause large primary and secondary follicle loss (Madden et al., under review). In addition, we have previously shown that these concentrations induce DNA damage and increased caspase 3 levels after 8 days of exposure. Our hypothesis was that altered Cx37 and Cx43 at timepoints prior to observed follicle loss may support that they are targets for DMBA as part of DMBA’s mechanism of ovotoxicity.

**Methods and Materials**

**Reagents**

7,12-Dimethylbenz[a]anthracene (DMBA; CAS # 57-97-6), bovine serum albumin (BSA), ascorbic acid, transferrin, 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N’N’N’N’-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, Sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco's Modified Eagle Medium:nutrient mixture F-12 (Ham) 1x (DMEM/Ham's F12), Albumax, penicillin (5000U/ml), Hanks' Balanced Salt Solution (without CaCl₂, MgCl₂ orMgSO₄) from Invitrogen Co. (Carlsbad, CA). Millicell-CM filter
inserts and 48 well cell culture plates were obtained from Millipore (Bedford, MA) and Corning Inc. (Corning, NY) respectively. RNeasy Mini kit, QIA shredder kit, RNeasy MinElute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

**Ovary culture**

Ovaries were collected from PND4 Fisher 344 female rats and cultured as described previously (Devine *et al*., 2002). Briefly, PND4 female F344 rat pups were euthanized by CO₂ inhalation followed by decapitation. Ovaries were removed, trimmed of oviduct and other excess tissues and placed onto membrane floating on 250 μl of DMEM/Ham's F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 μg/ml ascorbic acid, 5 U/ml penicillin and 27.5 μg/ml transferrin per well in a 48 well plate that had previously been equilibrated to 37 °C. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries were cultured for 4 days in control medium to allow development of large primary and secondary follicles, and were then treated once with medium containing vehicle control (1% DMSO) ± DMBA (12.5 nM or 75 nM) and the culture was maintained for four or eight days at 37 °C and 5% CO₂. This exposure induces pre-ovulatory follicle depletion after 8 days (Madden *et al*., under review). The medium was replaced every two days.
RNA isolation and qRT-PCR

RNA was isolated using an RNeasy Mini kit (Qiagen) and the concentration was determined using an ND-1000 Spectrophotometer ($\lambda = 260/280$nm; NanoDrop technologies, Inc., Wilmington, DE) ($n=3$; 10 ovaries per pool). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). cDNA was diluted (1:20) in RNase-free water. Diluted cDNA (2$\mu$l) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers for Cx37, Cx43 and Gapdh were designed by Primer 3 Input Version (0.4.0); Cx37 forward - tgatcagacctggtctgga; Cx37 reverse - aggagaatggtggtgtatg; Cx43 forward - gagcagttctcaacagtgc; Cx43 reverse - ccaacagcacaggttta: Gapdh forward - gttgacctgaatgccctcat; Gapdh reverse - ggtatgaattgtgagegaga: p53 forward - tgttacagcaatctcctat; p53 reverse - gatgtaggaatggtggtcct: Bax forward - caggtctatcagcactcatca; Bax reverse - ctaggcccttactcttccag. The regular cycling program consisted of a 15-min hold at 95°C and 45 cycles of denaturing at 95°C for 15s, annealing at 58°C for 15s, and extension at 72°C for 20s at which point data were acquired. There was no difference in Gapdh mRNA expression between treatments, thus each sample was normalized to Gapdh before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Protein isolation and Western blotting

Protein was isolated from cultured ovaries ($n=3$; 10 ovaries per pool). Homogenates were prepared from cultured ovaries via homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Thompson et al., 2005). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of
centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a BCA protocol. Protein was stored at -80 °C until further use. SDS-PAGE was used to separate protein homogenates which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 5 % milk in Tris-buffered saline containing tween 20, followed by incubation with anti-rabbit CX37 or CX43 antibody (1:100) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with species-specific secondary antibodies (1:2000) for 1 h at room temperature. Membranes were washed 3X in TTBS and incubated in chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values.

**Immunofluorescence Staining**

Ovaries were fixed in 4% paraformaldehyde for 2 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 µM thick), and every 10th section was mounted. Slides were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1M, pH 6.1). Sections were then blocked in 5% BSA for 1 h at room temperature. Sections were incubated with primary antibody CX37 (1:200) and CX43 (1:200) overnight at 4°C. After washing in 1% PBS, sections were incubated with the appropriate goat anti-rabbit IgG-FITC secondary antibodies for 1 h. Slides were then counterstained with 4-6-diamidino-2-phenylindole (DAPI) or Hoechst for 5 min. Images were taken using a Leica fluorescent microscope and protein expression were analyzed using ImageJ software (NCBI). 10-15 follicles per ovary (n=3) were analyzed for CX37 protein quantification and 3 ovaries were
used to quantify CX43 protein level by counting the number of CX43 positive foci in each ovary.

**Statistical analysis**

Raw data were analyzed by paired t-tests where two treatments were compared or ANOVA for comparison of more than two treatments, comparing treatment with control using Graphpad Prism 5.04 software. Values are expressed as mean ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05.

**Results**

**Effect of DMBA exposure on Cx37 and Cx43 mRNA expression**

DMBA exposure increased (P < 0.05) Cx37 mRNA (12.5 nM: 3.4-fold ± 0.9; 75 nM: 1.4-fold ± 0.9) at 4 days of exposure. In contrast, DMBA decreased (P < 0.05) Cx43 mRNA (12.5nM:0.5-fold ± 0.004; 75 nM: 0.7-fold ± 0.2), compared to vehicle-treated ovaries at 4 days of exposure (Figure 1A). At 8 days, both Cx37 and Cx43 mRNA expression were decreased (P < 0.05) by 12.5 nM (Cx37: 0.9-fold ± 0.007; Cx43: 0.85-fold ± 0.008) and 75 nM (Cx37: 0.95-fold ± 0.04; Cx43: 0.95-fold ± 0.06) DMBA compared to vehicle-treated ovaries (Figure 1B).

**DMBA alters CX37 and CX43 protein level**

CX37 protein level was increased (P < 0.05) by 12.5 nM (1.91 ± 0.006) and decreased (P < 0.05) by 75 nM DMBA (1.67 ± 0.05) at 4 days, relative to vehicle-treated ovaries (1.88 ± 0.06). CX43 protein level was increased (P < 0.05) by 12.5 nM (2.16 ± 0.03) and by 75 nM DMBA (1.62 ± 0.12) at 4 days, relative to vehicle-treated ovaries (0.84 ± 0.08) (Figure 2A). At 8 days, CX37 was decreased (P < 0.05) by 12.5 nM (0.95 ± 0.01) compared to vehicle-
treated ovaries (1.09 ± 0.02), but there was no impact of 75 nM DMBA on CX37. CX43 protein level was decreased \( (P < 0.05) \) by 12.5 nM (0.93 ± 0.02) and by 75 nM DMBA (0.78 ± 0.05) compared to vehicle-treated ovaries (1.01 ± 0.02) (Figure 2B).

**DMBA affects localization and amount of ovarian CX37 and CX43 proteins**

CX37 (Figure 3A-C) was localized to the oocyte cell surface. At 8 days, CX37 protein staining was increased \( (P < 0.05) \) in small primary follicles by 12.5 nM (45.58 ± 1.9) compared to 75 nM DMBA (34.35 ± 2.4) and vehicle-treated (37.33 ± 1.9) ovaries and in large primary and secondary follicles, CX37 was decreased \( (P < 0.05) \) by 12.5 nM (38.1 ± 0.73 and 31.04 ± 2.9) and by 75 nM (32.74 ± 0.7 and 21.01 ± 2.1) DMBA exposures, relative to vehicle-treated (43.17 ± 1.5 and 41.39 ± 1.7) ovaries, respectively (Figure 3H). CX43 (Figure 3D-F) was localized to the granulosa cells of all follicular stages. CX43 total protein staining was decreased \( (P < 0.05) \) by 12.5 nM (2708 ± 390) and 75 nM DMBA (3671 ± 1017) exposures compared to vehicle-treated (6648 ± 920) ovaries at 8 days (Figure 3G). In follicles that were devoid of oocytes, CX43 protein localization was absent or negligible.

**DMBA alters p53 and Bax mRNA level**

Relative to vehicle-treated ovaries, DMBA exposure decreased \( (P < 0.05) \) p53 mRNA (12.5 nM: 0.7-fold ± 0.04; 75 nM: 0.6-fold ± 0.1) after 4 days of exposure (Figure 4A). In contrast, DMBA increased \( (P < 0.05) \) p53 mRNA (12.5nM:0.6-fold ± 0.1; 75 nM: 0.4-fold ± 0.09), compared to vehicle-treated ovaries after 8 days of exposure (Figure 4B). DMBA exposure did not affect Bax mRNA (12.5 nM: 0.2-fold ± 0.2; 75 nM: 0.2-fold ± 0.2) after 4 days of exposure (Figure 4C). In contrast, DMBA increased \( (P < 0.05) \) Bax mRNA (12.5nM: 0.9-fold ± 0.08; 75 nM: 0.8-fold ± 0.1), compared to vehicle-treated ovaries after 8 days of exposure (Figure 4D)
Discussion

DMBA is an ovotoxicant that depletes follicles at various stages of development (Rajapaksa et al., 2007; Igawa et al., 2009; Madden et al., under review). The initiating event in DMBA-induced follicle destruction remains unclear; however, since cell to cell communication is vital for follicular viability, it is possible that perturbation to communication within the follicle could have detrimental consequences for the oocyte. CX37 and CX43 are the major ovarian gap junction proteins, shown to be essential for folliculogenesis and production of fertilizable oocytes (Granot et al., 2002; Kidder and Mhawi, 2002b; Teilmann, 2005). There is little information on whether ovotoxic chemicals target CX proteins and communication within the follicle, thus, we investigated whether DMBA had any impact on Cx37 and Cx43. Prior to DMBA-induced follicle loss, Cx37 mRNA and protein was increased and although Cx43 protein was elevated, mRNA encoding the Cx43 gene was decreased. These data indicate that DMBA may target Cx43 at the transcriptional level initially, and that increased Cx37 mRNA and protein and CX43 protein may be part of the ovarian protective response to DMBA exposure. Both Cx37 and Cx43 mRNA and protein were decreased by DMBA exposures at 8 days. This time coincides with DMBA-induced follicle depletion (Madden et al., under review) and therefore may simply be a consequence of reduced follicle number.

Cx37-null mice display retarded oocyte growth (Carabatsos et al., 2000) due to lack of nutrient intake from extracellular environment (Eppig, 1991). We found that CX37 protein was localized to the oocyte cell surface, in agreement with a previous report in the mouse ovary (Teilmann, 2005; Simon et al., 2006). Lack of organized CX37 localization around the oocyte perimeter has been reported as an early sign of follicular atresia (Teilmann, 2005),
thus, the perturbations to Cx37 mRNA and protein could impact follicular viability during DMBA-induced ovotoxicity. Interestingly, increased CX37 protein was evident in the small primary follicles at 12.5 nM DMBA. Since small primary follicle numbers are not impacted by DMBA at this concentration (Madden et al., under review), it is possible that increased CX37 is protective against DMBA-induced follicle loss.

Cx43 mRNA and protein level are correlated with follicular development in immature rats (Granot and Dekel, 1997). In extra-ovarian tissues, CX43 is inversely correlated with apoptosis, acting as a survival factor (Lin et al., 2003). The increase in Cx43 mRNA level after 4 days of DMBA exposure potentially indicates an anti-apoptotic role for ovarian CX43. Consistent with this posit, at the time point when follicle loss was observed (Madden et al., under review), Cx43 mRNA and protein were decreased by DMBA exposure which is consistent with lower expression of Cx43 mRNA and protein expression in cryopreserved apoptotic mouse ovarian tissue (Lee et al., 2008). This decrease in Cx43 mRNA and protein is also associated with increased caspase 3 protein in ovaries treated with the same DMBA concentrations (Ganesan et al., 2013). An elevated level of apoptosis associated with low expression of CX43 has also been reported in avian granulosa cells (Krysko et al., 2004). CX43 protein was localized between the granulosa cells in all stage follicles, consistent with a previous study that reported CX43 localization in the gap junctions connecting rat ovarian granulosa cells, with highest expression in pre-mature and pre-ovulatory antral follicles (Okuma et al., 1996; Gittens and Kidder, 2005). Interestingly, in large primary and secondary follicles that were devoid of oocytes, CX43 protein was either absent or negligible, implying that some oocyte-derived factor may be involved in CX43 protein maintenance. Despite our observed reduction of CX43 protein from total protein homogenates, the number of CX43
positive foci was increased. This may be representative of an attempt to increase gap junction communication and promote survival.

We also found increased levels of pro-apoptotic *p53* and *Bax* at the time of follicle loss (8 days) but not before the time of follicle loss (4 days). Decreased *p53* prior to follicle loss, could be due to interaction of *p53* with *mdm2*, resulting in *p53* inhibition (Lakin and Jackson, 1999). Additionally, there was no induction of *Bax* until the time-point at which follicle loss was observed (day 8). These results are in agreement with another study that demonstrated increased pro-apoptotic BAX and caspase-3 in granulosa cells of DMBA-exposed pre-ovulatory follicles (Tsai-Turton et al., 2007).

In summary, DMBA alters ovarian *Cx37* and *Cx43* at the transcriptional and post-transcriptional levels. The initial increase in CX37 and CX43 proteins in response to DMBA exposure demonstrates the capacity of ovarian tissue to mount a compensatory response to an ovotoxic insult.

**Conflict of Interest Statement**

The project described was supported by award number R00ES016818 to AFK and by a fellowship from the American Association of University Women to SG. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.

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Figure 1. Effect of DMBA exposure on Cx37 and Cx43 mRNA expression

Total RNA was isolated from PND4 rat ovaries that were cultured in control medium for 4 days followed by a single exposure to control (CT), 12.5 or 75 nM DMBA for (A) 4 or (B) 8 days. qRT-PCR was performed to amplify Cx37 or Cx43 mRNA. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = $P < 0.05$. DMBA alters connexins gene expression.
**Figure 2. Effect of DMBA exposure on CX37 and CX43 protein level**

Total protein was isolated from PND4 rat ovaries that were cultured in control medium for 4 days followed by a single exposure to control (CT), 12.5 or 75 nM DMBA for (A) 4 or (B) 8 days. (C) Western blotting was performed to quantify CX37 or CX43 protein level (day 8). Values are expressed as densitometric mean ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05. DMBA alters connexins protein levels.
Figure 3. Impact of DMBA exposure on ovarian CX37 and CX43 protein level

PND4 rat ovaries were cultured in control medium for 4 days followed by a single exposure to control (CT), 12.5 or 75 nM DMBA for 8 days. Ovarian sections were immunostained using primary (A-C) CX37 (green stain) or (D-F) CX43 (red stain) antibodies to check the localization and intensity of staining. Blue staining represents nuclear DNA staining. Thin arrows indicate primordial/small primary follicles; thick arrows indicate large primary/secondary follicles; chevron indicates follicle lacking an oocyte. ImageJ software was used to analyze the intensity of staining. 10-15 follicles per ovary (n=3) were used for (G) CX37 quantification and 3 ovaries were used for (H) CX43 positive foci quantification. Values are expressed as densitometric mean ± SE. *= P< 0.05; different from control.
Total RNA was isolated from PND4 rat ovaries that were cultured in control medium for 4 days followed by a single exposure to vehicle control (CT), 12.5 or 75 nM DMBA for (A, C) 4 or (B, D) 8 days. qRT-PCR was performed to amplify (A, B) p53 or (C, D) Bax mRNA. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05. DMBA increase apoptotic genes during follicle loss.
CHAPTER 5. IMPACT OF OBESITY ON 7,12-DIMETHYLBENZ[A]ANTHRACENE INDUCED ALTERED OVARIAN CONNEXIN GAP JUNCTION PROTEINS IN FEMALE MICE

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Contribution Statement:

I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Nteeba, J. aided with designing and performing the experiment and tissue collection. Keating, A.F. designed the experiments, aided in data interpretation and edited the manuscript.

Abstract

The ovarian gap junction proteins connexin 37, 43 and 45 are involved in cell communication and folliculogenesis. 7,12-dimethylbenz[a]anthracene (DMBA) alters CX37 and CX43 expression in cultured neonatal rat ovaries. Additionally, obesity has an additive effect on DMBA-induced ovotoxicity, thus, we investigated any impacts of obesity and DMBA on gap junction protein levels using an in vivo model. Ovaries were collected from lean and obese mice aged 6, 12, 18, or 24 wks. A subset of 18 wk old mice (lean and obese) was dosed with sesame oil or DMBA (1 mg/kg; i.p) for 14 days and ovaries collected 3 days thereafter. Cx43, Cx45 mRNA and protein levels were decreased (P < 0.05) after 18 wks while Cx37 mRNA and protein levels were decreased (P < 0.05) after 24 wks in obese ovaries. Cx37 mRNA and antral follicle protein staining intensity were reduced (P < 0.05) by obesity while total CX37 protein was reduced (P < 0.05) in DMBA exposed obese ovaries. Cx43 mRNA and total protein levels were decreased (P < 0.05) by DMBA in both lean and obese ovaries while basal protein staining intensity was reduced (P < 0.05) in obese controls. Cx45 mRNA, total protein and protein staining intensity level were decreased (P < 0.05) by
obesity. These data support that obesity temporally alters gap junction protein expression and that DMBA-induced ovotoxicity may involve reduced gap junction protein function.

Introduction

The ovary is the major female reproductive organ composed of follicles at different developmental stages from primordial to antral. Follicles contain a single oocyte, arrested in the diplotene stage of meiosis, which are surrounded by single to multiple layers of granulosa cells dependent on the stage of development. Granulosa cells are the somatic follicular cells and their functions include production of sex steroid hormones and growth factors which are essential for fertile reproductive life of women. Granulosa cell death by apoptosis is reportedly involved in the process of follicular atresia in the mammalian ovary (Tilly, 1997; Jiang et al., 2003).

Connexins (CX) are a family of transmembrane proteins that connect to form gap junctions; channels which allow direct exchange of ions and small molecules between adjacent cells and which are involved in cell proliferation, differentiation, cell survival, oocyte maturation, meiotic resumption and death (Gershon et al., 2008; Conti et al., 2012; Kar et al., 2012). Eight CX proteins are expressed in ovaries encoded by the Cx26, Cx30.3, Cx32, Cx37, Cx40, Cx43, Cx45 and Cx57 genes (Grazul-Bilska et al., 1997; Kidder and Mhawi, 2002). The most abundant gap junction proteins expressed in the mice ovaries are CX37, CX43 and CX45 (Simon et al., 1997; Ackert et al., 2001; Wright et al., 2001). CX37 is present between the oocyte and granulosa cell (Simon et al., 1997) while CX43 (Valdimarsson et al., 1993; Gittens, 2003) and CX45 (Okuma et al., 1996; Alcoléa et al., 1999; Wright et al., 2001) co-localize between granulosa cells. CX proteins are expressed in
a temporal pattern during follicular development and maturation in mouse ovaries (Wright et al., 2001), and defects in oocyte and follicular development have been identified in CX37 deficient mice in which heterologous oocyte-granulosa cell gap junctions were underdeveloped (Carabatsos et al., 2000). CX43 protein expression was also reduced in ovarian granulosa cells during follicular atresia in pigs and swamp buffaloes (Cheng et al., 2005; Feranil et al., 2005). These studies indicate that CXs are important for completion of oocyte growth, acquisition of cytoplasmic meiotic competence and follicular survivability.

CX proteins are targeted by chemicals including retinoids, carotinoids, chemotherapeutic agents (King and Bertram, 2005), cigarette components (McKarns et al., 2000) and polycyclic aromatic hydrocarbons including 7,12-dimethylbenz[a]anthracene (DMBA) (Sharovskaya et al., 2006; Ganesan and Keating, 2014). DMBA, liberated from burning of organic matter incineration, destroys all type of follicles in the ovaries of exposed mice and rats leading to ovarian failure (Gelboin, 1980; Mattison and Schulman, 1980). Cigarette smoke causes premature menopause onset in female smokers compared to their age matched non-smoking counterparts (Mattison et al., 1983; Alcoléa et al., 1999; Harlow and Signorello, 2000) and the offspring of female smokers have decreased numbers of oocytes, potentially leading to infertility (Jurisicova et al., 2007). DMBA destroys follicles by inducing apoptosis through increased expression of pro-apoptotic BAX and activation of the executioner protein caspase 3 (Tilly et al., 1991; Tsai-Turton et al., 2007). Additionally, DMBA alters the connexin gap junction protein expression when neonatal cultured rat ovaries were exposed in vitro (Ganesan and Keating, 2014).

Approximately one-third of adults in the USA are obese (Flegal Km et al., 2010; Meeker et al., 2010), and negative female phenotypic associations include polycystic ovarian
syndrome, menstrual disorders, intrauterine fetal death and infertility (Haslam and James; Cardozo et al., 2012). Obesity also detrimentally affects pregnancy rates in natural and assisted conception potentially by reducing oocyte quality (Wu et al., 2011). Primordial and small primary follicle numbers were reduced in ovaries from obese mice with a concomitant increase in number of secondary and pre-ovulatory follicles relative to lean mouse ovaries (Nteeba et al., 2014a). Additionally, ovaries from mice fed a high fat diet showed increased accumulation of endoplasmic reticulum stress, decreased mitochondrial activity and increased apoptosis of cumulus oocyte complexes and ovarian cells (Wu et al., 2010). Interestingly, a high fat diet also reduced cardiovascular connexin expression in female rats resulting in increased risk of ventricular arrhythmia (Aubin et al., 2010). Taken together, both DMBA and obesity have separately been shown to affect Cx gene mRNA and protein levels, thus this study investigated both the impact of obesity and DMBA exposure on ovarian Cx mRNA and protein levels using the lethal yellow mouse model of progressive obesity.

Methods and Materials

Reagents

7,12-dimethylbenz[a]anthracene (DMBA; CAS # 57-97-6), sesame oil (CAS # 8008-74-0), 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N’N’N’-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, Sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State
University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

**Animals**

The ovarian tissues utilized in this study were obtained as part of a larger study in our laboratory (Nteeba et al., 2014a; Nteeba et al., 2014b). Briefly, four week old female wild type normal non-agouti (a/a; designated lean; n = 10) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 10) were purchased from Jackson laboratories (Bar Harbor, ME 002468). All animals were housed in cages under a 12 h light/dark photoperiod with the temperature between 70-73°F and humidity approximately 20-30%. The animals were provided with a standard diet (Teklad 2014 global 14% protein rodent maintenance diet) with *ad libitum* access to food and water until 6, 12, 18 or 24 wks of age. Also, a subset of 18 wk old mice were dosed with sesame oil (vehicle control; n = 5 lean; n = 5 obese) or DMBA (1mg/kg; intraperitoneal injection; n = 5 lean; n = 5 obese) for 14 days and ovaries collected 3 days after the end of exposure. This DMBA dose was chosen based on the literature to induce approximately 50% primordial follicle loss (Borman et al., 2000). All procedures were approved by the Iowa State University Animal Care and Use Committee.

**Tissue collection**

At the end of each experimental time point, mice were euthanized in their pro-estrus phase of cyclicity by CO₂ asphyxiation. Ovaries were collected and cleaned off excess fat and stored in RNALater at -80°C or fixed in 4% paraformaldehyde.
RNA isolation and qRT-PCR

RNA was isolated using an RNeasy Mini kit (Qiagen) and the concentration determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE) (n=3). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). Diluted (1:20) cDNA (2 μl) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers for Cx37, Cx43 and Cx45 and Gapdh were designed by Primer 3 Input Version (0.4.0) (Table 3). The regular cycling program consisted of a 15 min hold at 95°C and 45 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 20 s at which point data were acquired. There was no difference in Gapdh mRNA expression between treatments, thus each sample was normalized to Gapdh before quantification. Quantification of fold-change in gene expression was performed using the $2^{ΔΔCt}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Protein isolation and western blotting

Protein was isolated from whole ovaries (n=3) by homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Ganesan and Keating, 2014). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a standard BCA protocol. SDS-PAGE was used to separate protein homogenates which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 5 % milk in Tris-buffered saline containing tween 20, followed by incubation in anti-rabbit CX37, CX43 and CX45 primary antibodies (1:100) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with species-specific
secondary antibodies (1:3000) for 1 h at room temperature. Membranes were washed 3X in TTBS and incubated in enhanced chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values.

**Immunofluorescence staining**

Ovaries were fixed in 4% paraformaldehyde for 2 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 µM thickness), and every 10th section was mounted. Slides were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1M, pH 6.1). Sections were then blocked in 5% BSA for 1 h at room temperature. Sections were incubated with primary antibody directed against CX37 (1:200), CX43 (1:200) or CX45 (1:100) overnight at 4°C. After washing in 1% PBS, sections were incubated with the appropriate goat anti-rabbit IgG-FITC secondary antibody for 1 h. Slides were then counterstained with 4-6-diamidino-2-phenylindole (DAPI) for 5 min. Images were taken using a Leica fluorescent microscope and protein expression were analyzed using ImageJ software (NCBI). 10 antral follicles per ovary and 3 ovaries were used for protein analysis.

**Statistical analysis**

Raw data were analyzed by unpaired t-test using Graphpad Prism 5.04 software. Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for total protein and protein, n=3. Different letters or asterisk (*) indicate statistical significance at $P < 0.05$. 
Results

Effect of progressive obesity on Cx37 mRNA and protein abundance

In lean mice, Cx37 mRNA levels were increased ($P < 0.05$) at 24 wks compared to earlier time points (Figure 1A) while no changes in obese ovaries across time points (Figure 1B). At 24 wks of age, obese mice had lower ($P < 0.05$) ovarian Cx37 mRNA abundance relative to their lean counterparts (Figure 1C). CX37 protein levels were decreased ($P < 0.05$) in ovaries from lean mice at both 18 and 24 wks, relative to 6 and 12 wks of age, however, the level of CX37 was greater ($P < 0.05$) at 24 wks than 18 wks (Figure 1D). A similar pattern was observed in CX37 protein in obese ovaries; however the 24 wk rebound did not occur (Figure 1E). Comparison of lean to obese ovarian CX37 levels revealed that obese ovaries had higher ($P < 0.05$) CX37 levels at 12 wks but lower ($P < 0.05$) levels at 24 wks, relative to lean ovaries (Figure 1F, G).

Progressive obesity impact on Cx43 mRNA and protein level

A temporal pattern of increased ($P < 0.05$) Cx43 mRNA was observed in both lean and obese mice (Figure 2A, B). Comparison of Cx43 mRNA between ovaries from lean and obese mice demonstrated increased ($P < 0.05$) Cx43 mRNA at the 12 wk time point, however Cx43 mRNA was reduced ($P < 0.05$) in the 18 and 24 wk aged mice (Figure 2C) in obese relative to lean ovaries. Ovarian CX43 protein levels were increased ($P < 0.05$) over time in lean mice (Figure 2D). Progressive obesity resulted in reduced ($P < 0.05$) ovarian CX43 (Figure 2E) and this reduction was reflected in decreased ($P < 0.05$) CX43 in obese relative to lean ovaries at the 18 and 24 wks time points (Figure 2F,G).
Effect of obesity on ovarian Cx45 mRNA and protein abundance

There was a dramatic decrease ($P < 0.05$) in Cx45 mRNA over time in both lean and obese ovaries (Figures 3A, B). Interestingly, Cx45 mRNA abundance was greater ($P < 0.05$) in obese relative to lean ovaries at the 12 wk time point; however this effect was reversed ($P < 0.05$) at 18 and 24 wks of age (Figure 3C). CX45 protein level was decreased over time in both lean and obese ovaries (Figure 3D, E). In a similar manner to Cx45 mRNA, protein levels were greater ($P < 0.05$) in ovaries from obese mice at 12 wks of age, and a reduction ($P < 0.05$) was observed at both the 18 and 24 wk aged mice (Figure 3F, G).

DMBA effects on Cx37 mRNA and protein level

At the end of DMBA dosing and tissue collection, animals were 20.5 wks of age. Basal levels of Cx37 mRNA were decreased ($P < 0.05$) in obese compared to lean ovaries. In both lean and obese mice, levels of Cx37 mRNA were not changed by DMBA exposure compared to their control counterparts, however, Cx37 mRNA levels were decreased ($P < 0.05$) in obese relative to lean ovaries after DMBA exposure (Figure 4A). CX37 total protein basal levels were not changed in obese compared to lean ovaries. In lean mice, levels of CX37 total protein were not affected by DMBA exposure compared to control-treated ovaries. In contrast, DMBA exposure decreased ($P < 0.05$) CX37 total protein level in ovaries from obese mice, compared to obese control-treated animals (Figure 4B, C).

Impact of DMBA exposure on ovarian Cx43 mRNA and protein

Basal levels of Cx43 mRNA were not changed in obese compared to lean ovaries. In both lean and obese mice, levels of Cx43 mRNA were decreased ($P < 0.05$) by DMBA exposure compared to control-treated ovaries. Cx43 mRNA level was decreased to a greater extent ($P < 0.05$) in ovaries from obese mice relative to their lean littermates after DMBA
exposure (Figure 5A). CX43 total protein basal levels were not changed ($P < 0.05$) in obese compared to lean ovaries. In both lean and obese mice, levels of CX43 protein were decreased ($P < 0.05$) by DMBA exposure compared to control-treated ovaries. There was no difference in CX43 protein level between obese and lean ovaries after DMBA exposure (Figure 5B, C).

**Consequence of DMBA exposure on Cx45 mRNA and protein expression**

Basal levels of $C_x45$ mRNA were decreased ($P < 0.05$) in obese compared to lean ovaries. In both lean and obese mice, levels of $C_x45$ mRNA were not changed ($P < 0.05$) by DMBA exposure compared to their respective control-treated ovaries (Figure 6A). CX45 protein abundance was decreased ($P < 0.05$) in obese compared to lean ovaries. In lean mice, levels of CX45 total protein were not changed by DMBA exposure compared to control-treated ovaries. In contrast, DMBA exposure increased ($P < 0.05$) CX45 total protein level in ovaries from obese mice, compared to obese control-treated animals (Figure B, C).

**Localization and quantification of obesity and DMBA effects on CX proteins**

CX37 was localized to the oocyte cytoplasm of the oocyte and between the granulosa cells (Figure 7A-D). CX37 protein basal levels were lower ($P < 0.05$) in obese relative to lean ovaries. Similar to the western blotting data, in both lean and obese mice, levels of CX37 protein were unchanged by DMBA exposure compared to control-treated ovaries. CX37 protein level was decreased in obese ovaries compared to lean ovaries after DMBA exposure (Figure 7M). CX43 was localized between the granulosa cells (Figure 7E-H). CX43 protein levels were lower ($P < 0.05$) in antral follicles of obese relative to lean ovaries. In both lean and obese mice, levels of CX43 protein were decreased ($P < 0.05$) by DMBA exposure compared to control-treated ovaries. CX43 protein level was decreased to a greater
extent in obese ovaries compared to lean ovaries after DMBA exposure (Figure 7N). CX45 proteins were localized between the granulosa cells (Figure 7I-L). CX45 protein levels were lower \((P < 0.05)\) in obese relative to lean ovaries. In both lean and obese mice, levels of CX45 protein were unchanged by DMBA exposure compared to control-treated ovaries. CX45 protein level was lower in obese than in lean ovaries after DMBA exposure (Figure 7O).

**Discussion**

High fat diet fed mice have increased levels of apoptosis in cumulus oocyte complexes and granulosa cells (Wu et al., 2010) and reduced connexin expression in hearts of female rats (Aubin et al., 2010), potentially supporting that obesity alters connexin protein abundance. DMBA altered connexin gene expression in neonatal ovaries (Ganesan and Keating, 2014), however, the effect of DMBA on connexin level in adult mouse ovaries has not yet been studied. Also, our previous work has demonstrated that the level of an enzyme required for DMBA bioactivation is higher in ovaries of obese mice, potentially predisposing them to increased DMBA-induced ovotoxicity (Nteeba et al., 2014a). Thus, this study investigated the impacts of both progressive obesity and DMBA exposure on ovarian connexin mRNA and protein abundance.

CX37 is essential for follicular development, ovulation as well as luteal tissue growth, differentiation, and regression (Simon et al., 1997). Ovarian Cx37 mRNA level was increased in 24 wk old mice however total protein was decreased after 18 wks in lean mice ovaries potentially indicating that CX37 protein levels decline with ovarian aging. Cx37 mRNA was previously shown to be reduced in the mesenteric arteries of 25 wk old insulin
resistant obese compared to lean littermate control rats (Young et al., 2008). CX37 total protein was decreased after 18 wks in obese mouse ovaries. Ovaries from obese mice had lower levels of Cx37 mRNA and protein after 24 wks compared to lean ovaries, but in a similar manner to the lean mice, Cx37 mRNA declined with aging in obese females. This observed decline in Cx37 mRNA with progressive obesity could also be due to altered folliculogenesis as observed from 12 wks onwards (Nteeba et al., 2014b).

In a similar manner to our previous study in neonatal cultured rat ovaries (Ganesan and Keating, 2014), DMBA decreased CX37 total protein levels. Further, obesity had a greater impact on the DMBA-induced decrease in CX37; suggesting DMBA may accelerate the decrease in CX37 protein levels during obesity, further contributing to ovotoxicity. CX37 protein was localized in the cytoplasm of oocyte (oolemma) and in the granulosa cells of large follicles, consistent with our previous study (Ganesan and Keating, 2014) and others who showed that CX37 is present on the oocyte surface of pre-antral follicles and between the granulosa cells of large antral follicles in mouse ovaries (Wright et al., 2001; Teilmann, 2005; Simon et al., 2006). Loss of organized CX37 localization around the oocyte perimeter is an early sign of follicular atresia (Teilmann, 2005), and suggests that intact cellular communication between the oocyte and the somatic cells is mandatory for follicular health. CX37 antral follicle protein staining intensity was reduced in both the obese control and DMBA treated ovaries compared to lean ovaries, in agreement with our previous work demonstrating that CX37 protein was decreased in cultured neonatal rat ovaries after DMBA exposure (Ganesan and Keating, 2014). Reduced CX37 has also been shown in denuded oocytes of diabetic mice compared to non-diabetic mice (Ratchford et al., 2008). Our results indicate that CX37 protein levels are reduced by aging, progressive obesity and DMBA
exposure, potentially interfering with the role of CX37 during follicular development, maintenance of germinal vesicle and ovulation.

CX43 forms channels between granulosa cells, which are required for their proliferation (Gittens, 2003). Granulosa cells recovered from Cx43<sup>−/−</sup> mice fail to show electrical coupling (Tong <i>et al.</i>, 2005). In the present study, Cx43 mRNA and protein levels were increased in lean ovaries after 18 wks which was similar to previous study that CX43 protein expression was increased in wild-type littermates compared to Gja1<sup>Int/−</sup> mutant mice after 11 wks of age in ovaries (Flenniken <i>et al.</i>, 2005). However, Cx43 mRNA and protein levels were decreased in obese ovaries compared to lean ovaries in agreement with another study demonstrating that a high fat diet induced low CX43 protein levels, restorable by pharmacological intervention in cardiac tissues of 32 wk old mice (Noyan-Ashraf <i>et al.</i>, 2013). Decreased CX43 levels in autopsied uterine tissue were also demonstrated in rats fed a high fat and high cholesterol diet for approximately 8.5 wks (Elmes <i>et al.</i>, 2011). These data further support that CX43 protein levels are decreased during progressive obesity.

DMBA exposure decreased Cx43 mRNA and total protein levels both in lean and obese mice ovaries consistent with our previous study in neonatal female rat pups ovaries which indicated that DMBA reduced Cx43 mRNA and protein levels prior to follicle loss (Ganesan and Keating, 2014). Since DMBA is a component of cigarette smoke these results are consistent with previous studies that cigarette smoke reduced CX43 expression in the corporal cavernosum of male rats (Liu <i>et al.</i>, 2011) and in human pancreatic ductal epithelial cells (Tai <i>et al.</i>, 2007). Cx43 mRNA and protein were also decreased in atretic follicles in rat ovaries (Wiesen and Midgley, 1994). Immunohistochemistry staining in our study localized CX43 protein between the granulosa cells and CX43 protein staining intensity was reduced
in obese ovaries. The intensity of CX43 protein staining was also reduced by DMBA exposure in lean and obese ovaries. An increase in apoptotic follicles associated with a decrease in the ovarian CX43 expression was recently shown in acute hyperglycemia and chronic diabetic female mice (Chang et al., 2005). The reduced amount of CX43 protein staining by DMBA exposure and by obesity indicates that antral follicles could be undergoing atresia.

CX45 is localized between granulosa cells and expressed throughout follicular development (Okuma et al., 1996). Cx45 mRNA and protein were decreased in both lean and obese ovaries after 12 wks of age but CX45 protein increased after 18 wks of age in lean ovaries. In contrast, the level of Cx45 mRNA and protein were both reduced after 18 wks of age in obese relative to lean ovaries. Also, Cx45 mRNA and total protein levels were reduced in obese ovaries compared to lean ovaries after DMBA exposure. Since this is the first study to explore the level of CX45 protein levels after chemical exposure, the results indicate that CX45 is responsive to both DMBA exposure and progressive obesity. CX45 staining was found to be restricted to gap junctions between granulosa cells of large antral follicles (Wright et al., 2001). We also found that CX45 protein was present between the granulosa cells of antral follicles and the intensity of protein staining was reduced by obesity in antral follicles of obese mice. Additionally, CX45 is reported to co-localize with CX43 protein in rat ovaries (Okuma et al., 1996). Our results suggest that both CX43 and CX45 protein staining intensity were reduced by obesity and that CX43 is more sensitive to DMBA exposure than CX45.
In summary, we report that ovarian aging, obesity and DMBA exposure each alter connexin gap junction protein expression in ovaries in a manner that could contribute to compromised reproduction observed during each one of these physiological paradigms.

Acknowledgement

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References


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smoke condensates from cigarettes that burn or primarily heat tobacco. Toxicology in Vitro 14, 41-51.


Table 1. Primer sequences used for qRT-PCR

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<th>Gene</th>
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Figure 1. Effect of progressive obesity on Cx37 mRNA and protein abundance

Ovaries were collected from 6, 12, 18 or 24 wks aged mice to isolate RNA and protein to perform qRT-PCR (A-C) and western blot (D-G). Analysis between lean alone (A, D), obese alone (B, E) and between lean and obese or interaction (C, F) and values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for protein expression; n=3. Different letters or asterisk (*) indicate the statistical significance P < 0.05. Connexin 37 decreases during aging and during progressive obesity.
Figure 2. Progressive obesity impact on Cx43 mRNA and protein level

Ovaries were collected from 6, 12, 18 and 24 wks of age to isolate RNA and protein to perform qRT-PCR (A-C) and western blot (D-G). Analysis between lean alone (A, D), obese alone (B, E) and between lean and obese or interaction (C, F) and values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for protein expression; n=3. Different letters or asterisk (*) indicate the statistical significance $P < 0.05$. Connexin 43 increases after 24 wks while decreases by progressive obesity.
Figure 3. Effect of obesity on ovarian Cx45 mRNA and protein abundance

Ovaries were collected from 6, 12, 18 and 24 wks of age to isolate RNA and protein to perform qRT-PCR (A-C) and western blot (D-G). Analysis between lean alone (A, D), obese alone (B, E) and between lean and obese interaction (C, F) and values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for protein expression; n=3. Different letters or asterisk (*) indicate statistical significance $P < 0.05$. Connexin 45 increases after 24 wks while decreases by progressive obesity.
Figure 4. DMBA effects on Cx37 mRNA and protein level

Following exposure to DMBA at 1mg/kg for 14 days, total mRNA and protein were isolated to perform qRT-PCR (A) and western blot analysis (B, C). Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for total protein; n=3. Different letters indicate the statistical significance $P < 0.05$. DMBA alters CX 37 protein during obesity.
Figure 5. Impact of DMBA exposure on ovarian Cx43 mRNA and protein

Following exposure of DMBA at 1mg/kg for 14 days, total mRNA and protein were isolated to perform qRT-PCR (A) and western blot analysis (B, C). Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for total protein; n=3. Different letters indicate the statistical significance $P < 0.05$. DMBA reduces CX 43 proteins in both lean and obese ovaries.
Figure 6. Consequence of DMBA exposure on Cx45 mRNA and protein expression

Following exposure of DMBA at 1mg/kg for 14 days, total mRNA and protein were isolated to perform qRT-PCR (A) and western blot (B,C) analysis. Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for total protein; n=3. Different letters indicate the statistical significance $P < 0.05$. Obesity reduces CX 45 protein levels compared to lean ovaries.
Following exposure of DMBA at 1mg/kg for 14 days, paraffin embedded ovarian sections were immunostained using primary CX37 (A-D), CX43(E-H) and CX45 (I-L) antibodies to check the localization and intensity of staining for CX37 (M); CX43 (N) and CX45 (O). ImageJ software was used to analyze the intensity of staining (M-O). 10-large follicles per ovary and 3 ovaries were used for protein analysis. Values are expressed as mean ± SE. Different letters indicate the statistical significance $P < 0.05$. CX37 and CX45 protein staining decreases by obesity while CX 43 decreases by DMBA.
CHAPTER 6. PHOSPHORAMIDE MUSTARD-INDUCED DNA ADDUCT FORMATION AND DAMAGE REPAIR RESPONSE IN RAT OVARIAN GRANULOSA CELLS

A paper submitted to Toxicology and Applied Pharmacology

Shanthi Ganesan and Aileen F. Keating

Contribution Statement:
I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Aileen F. Keating designed the experiments, aided in data interpretation and edited the manuscript.

Abstract

Phosphoramide mustard (PM), the ovotoxic metabolite of the anti-cancer agent cyclophosphamide (CPA), destroys rapidly dividing cells by forming NOR-G-OH, NOR-G and G-NOR-G adducts with DNA, potentially leading to DNA damage. A previous study demonstrated that PM induces ovarian DNA damage in rat ovaries. To investigate whether PM induces DNA adduct formation, DNA damage and induction of the DNA repair response, rat spontaneously immortalized granulosa cells (SIGC) were treated with vehicle control (1% DMSO) or PM (3 or 6 µM) for 24 or 48 h. Cell viability was reduced ($P < 0.05$) after 48 h of exposure to 3 or 6 µM PM. The NOR-G-OH DNA adduct was detected after 24 h of 6 µM PM exposure, while the more cytotoxic G-NOR-G DNA adduct was formed after 48 h by exposure to both PM concentrations. Phosphorylated H2AX ($\gamma$H2AX), a marker of DNA double stranded break occurrence, was also increased by PM exposure, coincident with DNA adduct formation. Additionally, induction of genes and proteins involved in DNA repair were observed in both a time- and dose-dependent manner. These data support that PM induces DNA adduct formation in ovarian granulosa cells, induces DNA damage and elicits the ovarian DNA repair response.
Introduction

Infertility is a side effect of female cancer treatment. Female cancer treatment survivors have reduced fecundity, relative to their non-treated counterparts (Hudson, 2010). In addition, cancer treatment can reduce the ovarian follicular reserve, resulting in premature ovarian failure (POF) (Hudson, 2010). Fewer primordial follicles have been demonstrated in autopsied ovarian samples (Himelstein-Braw et al., 1977) and fewer antral follicles detected by ultrasound (Larsen et al., 2003) in females that received anti-neoplastic treatment compared to their age-matched control subjects.

Cyclophosphamide (CPA) is an alkylating agent used to treat both cancer and autoimmune disorders. CPA causes acute ovarian failure in childhood cancer survivors (Chemaitilly et al., 2006) and rapid amenorrhea in women undergoing adjuvant treatment for breast cancer (Minton and Munster, 2002). CPA is a pro-drug requiring hepatic biotransformation by cytochrome P-450 enzymes, to generate an active cytotoxic metabolite, phosphoramide mustard (PM) (Plowchalk and Mattison, 1991; Madden et al., 2014). PM is the anti-neoplastic as well as the ovotoxic metabolite of CPA (Desmeules and Devine, 2006; Petrillo et al., 2011). PM causes primordial and small primary follicle loss (Petrillo et al., 2011) and a volatile metabolite of PM, chloroethylaziridine (CEZ) is also likely involved in PM-induced ovotoxicity (Madden et al., 2014). PM induces DNA or chromosomal damage in mammalian cells, important genotoxicity markers (Anderson et al., 1995). PM is also an alkylating agent, known to cause cytotoxicity though forming cross-linked DNA adducts which inhibit DNA strand separation during replication (Phillips et al., 2000). DNA alkylation by PM occurs primarily at the N-7 position of guanine (Mehta et al., 1980) giving rise to the first product, a guanosine-PM adduct, which is unstable with a half-life of ~2-3 h
PM forms a mono-functional DNA adduct; N-(2-chloroethyl)-N-[2-(7-guaninyl)ethyl]amine (NOR-G) and a bi-functional DNA adduct; N-(2-hydroxyethyl)-N-[2-(N7-guaninyl)ethyl]-amine (NOR-G-OH) and N,N-bis[2-(N7-guaninyl)ethyl]amine (G-NOR-G), DNA adduct both *in vitro* (Cushnir et al., 1990) and *in vivo* (Malayappan et al., 2010) (Figure 1). The cross-linked adduct G-NOR-G is responsible for the cytotoxicity and teratological effects of CPA/PM (Little and Mirkes, 1987). PM has been shown to induce DNA damage in exposed ovaries of mice and rats (Petrillo et al., 2011).

Upon DSB induction, cells activate DNA damage responses (DDR) that comprise of cell cycle arrest, DNA damage repair, and subsequent cell cycle resumption or cell death (Giunta et al., 2010). One of the most immediate DDR events is phosphorylation of histone H2AX (γH2AX), considered the gold standard for localizing DSBs since it recruits and maintains DNA repair molecules at damage sites until repair is completed (Svetlova et al., 2010). Some DDR proteins activated due to DSBs include ataxia-telangiectasia mutated (ATM), ATM related (ATR) and DNA-dependent protein kinases (DNA-PKcs) (Svetlova et al., 2010). DNA DSBs can be repaired by both the non-homologous end joining (NHEJ) and homologous recombination (HR) pathways.

The granulosa cell is the somatic cell component of the oocyte-containing follicle, and close association between the granulosa cell and oocyte is required for follicular development. Some functions of granulosa cells include the production of sex steroids (Bjersing and Carstensen, 1967) and a myriad of growth factors that interact with the oocyte during development (Forde et al., 2008). Loss of granulosa cells during pre-antral and antral stages of follicular development leads to a premature reduction in female fecundity through reduced follicle health and oocyte viability (Walters et al., 2012). An *in vivo* study
demonstrated that the destruction of granulosa cells by CPA potentially occurs through apoptosis in rats (Lopez and Luderer, 2004). Since PM-induced DNA damage could predispose the follicle towards atresia, this study investigated whether PM exposure to granulosa cells results in PM-DNA adducts formation and subsequent DDR induction.

Methods and Materials

Reagents

Phosphoramide mustard (PM) was obtained from the National Cancer Institute (Bethesda, MD). 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, Ammonium persulphate, Glycerol, N‘N‘N‘N‘-Tetramethylethylenediamine (TEMED), Tris base, Tris HCl, Sodium chloride, Tween-20, Bis- 2-chloroethylamine hydrochloride, 2′-deoxyguanosine and sodium acetate were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco’s Modified Eagle Medium (D-MEM)/F-12 (1x), 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA), Pen Strep and Fetal Bovine Serum (FBS) from Gibco by Life Technologies (Grand Island, NY). Millicell-EZ slides were from Millipore (Bedford, MA). Corning Vacuum Filter/Storage System and cell culture flasks were purchased from Corning Inc. (Corning, NY). RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, QuantitectTM SYBR Green PCR kit and Blood and cell culture DNA mini kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). The polyclonal goat anti-rabbit secondary were obtained from Pierce Biotechnology (Rockford, IL). Ponceau S was from Fisher Scientific.
Rat spontaneously immortalized granulosa cell culture

A spontaneously immortalized clonal granulosa cell line (SIGC) derived from primary rat ovarian granulosa cell cultures were obtained as a gift from Dr. Burghardt at Texas A&M University. SIGC cells (2.5×10^4 cells) were cultured in 25-cm² flasks in media (DMEM/F12 plus 5% FBS and 50mg/ml of Pen Strep) at 37°C and 5% CO₂ until 80% confluent.

**Cell viability**

SIGC cells were treated with DMSO and/or PM (0.5 µM, 1 µM, 3 µM or 6µM) for 48 h to perform cell viability measurements. Cells were harvested by trypsinization and 100 µl of the cell suspension was stained with Trypan blue (1:1). Trypan blue selectively penetrates cell membranes of dead cells, coloring them blue, whereas it is not absorbed by membranes of live cells, thus excluding live cells from staining. Cell counting was performed using a hemocytometer and the percentage of viable cells calculated.

**DNA isolation and mass spectrometry**

Cells were harvested by trypsinization and pelleted by centrifugation at 1200 RPM for 5 min, washed twice in phosphate buffer saline (PBS) and resuspended in cold PBS (4°C). DNA was isolated from cells using a Qiagen blood and cell culture DNA kit according to the manufacturer’s protocol. DNA concentration was determined using NanoDrop (λ = 260/280 nm; ND 1000; Nanodrop technologies Inc., Wilmington, DE). Internal standards of G-NOR-G and NOR-G-OH were prepared according to a modified procedure (Hemminki, 1985; Malayappan *et al.*, 2010). DNA samples from cell lines (10-50 μg in 200 μL of buffer) were used to perform Agilent QTOF6540 LC/MS analysis at the Iowa State University Chemical Instrumentation Facility. Each sample (1 µl) was injected into the Jet stream ESI ion source and water/MeOH (20/80) was used as effluent solvent. The LC column used in this
experiment was C18, 4.6×150 mm, 1.8 μm and accurate mass measurement was achieved by constantly infusing a calibrant (masses: 121.0508 and 922.0098).

**Immunofluorescence**

Cells (1 x 10^5 cells/well) were transferred to a Millicell-EZ slide wells (Millipore, Bedford, MA) and treated with DMSO ± PM (3 or 6 μM) for 24 or 48 h. Cells were washed with PBS three times and fixed with 95% ethanol for 5 min on ice, followed by incubation in 0.25-0.5% Triton X-100 containing PBS for 10 minutes to permeabilize the membranes. After washing, slides were blocked with 5% normal serum in PBS for 1 h and washed again with PBS. Slides were incubated with a primary antibody directed against γH2AX (1:50) overnight at 4°C. After washing in PBS, slides were incubated with a donkey anti-rabbit IgG-FITC secondary antibody (1:3000) for 1 h. Slides were then counterstained with 4-6-diamidino-2-phenylindole (DAPI). Images were taken using a Leica fluorescent microscope and the percentage of cells with foci for γH2AX was calculated (n=3 slides/treatment).

**RNA isolation and qRT-PCR**

SIGC cells were treated with DMSO ± PM (3 or 6μM) for 24 or 48 h. RNA was isolated using an RNeasy Mini kit and the concentration was determined using a NanoDrop (λ = 260/280 nm; ND 1000; Nanodrop technologies Inc., Wilmington, DE). Total RNA (1 μg) was reverse transcribed to cDNA utilizing the Superscript III One-Step qRT-PCR. Diluted cDNA (2 μl; 1:20) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit. Primers for *Atm*, *Prkdc*, *Xrcc6*, *Parp1*, *Rad51*, *Brcal* and *Gapdh* were designed by Primer 3 Input Version (0.4.0) (Table 3). The regular cycling program consisted of a 15 min hold at 95°C and 45 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 20 s at which point data were acquired.
Each sample was normalized to *Gapdh* before quantification using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001)

**Protein isolation and western blot**

SIGC cells were treated with DMSO ± PM (3 or 6 µM) for 24 or 48 h and protein isolated using lysis buffer containing protease and phosphatase inhibitors as previously described (Ganesan *et al.*, 2013). Briefly, cells were placed on ice for 10 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a BCA protocol. Protein was stored at -80 °C until further use. SDS-PAGE was used to separate the proteins and then transferred to nitrocellulose membrane. Membranes were blocked for 1 h in 5 % milk in Tris-buffered saline containing tween 20. Membrane were incubated in one of: anti-PARP1 antibody (1:200), anti-ATM antibody (1:100), anti-rabbit phosphorylated H2AX antibody (γH2AX; 1:100), anti-rabbit BRCA1 antibody (1:500), anti-rabbit PRKDC antibody (1:100), anti-RAD 51 antibody (1:500) and anti-mouse XRCC6 antibody (1:100) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with a species specific secondary antibody (1:2000) for 1 h at room temperature. Membrane were washed three times in TTBS and one time in TBS and then incubated in enhanced chemiluminescence (ECL) detection substrate for 5 min and exposed to X-ray film. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). All proteins were normalized to Ponceau S densitometry values.

**Statistical analysis**

Data were analyzed by unpaired t-tests comparing treatment with control raw data at each individual time-point using Prism 5.04 software (Graph Pad Software). Statistical
significance was defined as $P<0.05$. For graphical purposes, protein expression is presented as a mean ± SE of the respective control.

**Results**

**Cell viability was impacted by PM exposure**

SIGC cells were treated with DMSO or PM (0.5, 1, 3 or 6 µM) for 48 h and cell viability quantified using Trypan blue staining. PM reduced ($P < 0.05$) cell viability at concentrations of 3 µM and higher. Relative to control, the percentage of viable cells was 91% and 83.6% after exposure to 3 µM and 6 µM PM, respectively (Figure 2).

**PM exposure induces DNA adduct formation**

SIGC cells were treated with DMSO or PM (3 or 6 µM) for 12, 24 or 48 h and LC/MS analysis performed. A NOR-G-OH (Figure 3A, B) DNA adduct was observed after 24 h of exposure to 6 µM PM, while the G-NOR-G (Figure 3C, D) DNA adduct was formed after 48 h with both the 3 and 6 µM PM exposures.

**DNA damage was induced by PM exposure as determined by localization of γH2AX**

SIGC cells were treated with DMSO or PM (3 or 6 µM) for 24 or 48 h to perform γH2AX protein localization (Figure 4A-C) using immunofluorescence. The percentage of cells with positive γH2AX foci were increased ($P < 0.05$) by the 6 µM PM exposure after 24 h, relative to control treated cells. After 48 h of PM exposure (both concentrations) increased levels of γH2AX foci were observed (Figure 4D).

**PM exposure increases DDR gene mRNA expression level**

Following 24 h of PM exposure, the DDR genes *Atm* (2.2 fold ± 0.5), *Parp1* (2.2 fold ± 0.4), *Prkdc* (2.2 fold ± 0.2), *Xrcc6* (2.1 fold ± 0.4), *Brcal* (2.0 fold ± 0.3) and *Rad51* (3.3 fold ± 0.7) were significantly upregulated compared to control.
fold ± 0.7) mRNA expression were increased ($P < 0.05$) after 6 µM concentration compared to both control and cells exposed to 3 µM PM. *Atm* (0.5 fold ± 0.09), *Parp1* (0.4 fold ± 0.01), *Prkdc* (0.4 fold ± 0.07), *Xrcc6* (0.4 fold ± 0.06), *Brca1* (0.6 fold ± 0.07) and *Rad51* (0.4 fold ± 0.04) mRNA expression were decreased ($P < 0.05$) by 3 µM PM exposure after 24 h compared to both control and 6 µM PM treated cells (Figure 5A).

After PM exposure for 48 h, mRNA abundance of the DNA repair response genes *Atm* (3.1 fold ± 0.6), *Parp1* (4.9 fold ± 1.1), *Prkdc* (4.1 fold ± 0.6), *Brca1* (3.4 fold ± 0.4) and *Rad51* (8.6 fold ± 2.4) mRNA expression were increased ($P < 0.05$) at 3 µM concentration while no changes in *Xrcc6* (1.5 fold ± 0.4) mRNA expression were observed compared to control and 6 µM PM. At the same time *Atm* (2.3 fold ± 0.3), *Parp1* (2.2 fold ± 0.4), *Brca1* (2.1 fold ± 0.6) were increased at the 6 µM PM concentration compared to control and 3 µM PM. No changes were detected in *Prkdc* (1.4 fold ± 0.3), *Xrcc6* (0.4 fold ± 0.01), or *Rad51* (1.4 fold ± 0.3) mRNA expression at 3 µM compared to control and 6 µM PM exposure (Figure 5B).

**DDR proteins were increased in response to PM exposure**

Following 24 h of PM exposure, the DDR proteins γH2AX (0.8 ± 0.03), ATM (1.05 ± 0.07), PRKDC (0.9 ± 0.02), XRCC6 (1.2 ± 0.02) and RAD51 (1.2 ± 0.01) were increased ($P < 0.05$) by exposure to 6µM PM. BRCA1 (3 µM: 1.3 ± 0.009; 6 µM: 1.5 ± 0.03) and PARP1 (3 µM: 1.2 ± 0.02; 6µM: 1.4 ± 0.09) were increased ($P < 0.05$) by both concentrations of PM compared to control. In contrast, PRKDC (0.8 ± 0.01) and RAD51 (0.9 ± 0.02) were decreased ($P < 0.05$) by 3 µM PM compared to both control and 6 µM PM treatments (Figure 6A).
After 48 h of PM exposure, the DDR proteins γH2AX (3 µM: 0.4 ± 0.006; 6 µM: 0.4 ± 0.007), ATM (3 µM: 4 ± 0.02; 6 µM: 0.4 ± 0.009), PARP1 (3 µM: 0.8 ± 0.01; 6 µM: 0.7 ± 0.03), PRKDC (3 µM: 0.8 ± 0.01; 6 µM: 0.7 ± 0.03) and BRCA1 (3 µM: 0.9 ± 0.01; 6 µM: 0.9 ± 0.03) were increased ($P < 0.05$) at both PM concentrations compared to control while XRCC6 (3 µM: 0.9 ± 0.04; 6 µM: 0.8 ± 0.03) protein was decreased ($P < 0.05$) by 6 µM, but not 3 µM, PM concentration. RAD51 (3 µM: 0.7 ± 0.01; 6 µM: 0.8 ± 0.05) protein was not altered by either concentration of PM compared to control (Figure 6B).

**Discussion**

CPA has been reported to destroy ovarian follicles by targeting granulosa cells in mice and rats (Desmeules and Devine, 2006). PM-induced DNA damage not only presents in the nucleus of oocyte and also occurs in granulosa cells (Petrillo et al., 2011). Since PM induces DNA damage in granulosa cells, we assessed whether DNA adduct formation occurs and if there is a subsequent DDR due to PM exposure in the rat ovarian SIGC line. It is known that the cytotoxic properties of PM occur by formation of DNA adducts, thereby preventing cell division by inhibiting DNA strand separation (Cushnir et al., 1990). In addition, the cross-linked adduct G-NOR-G is responsible for the cytotoxicity and teratological effects of CPA (Little and Mirkes, 1987) and these events are required for tumor destruction. PM formed the G-NOR-G adduct in exposed rat embryos (Mirkes et al., 1992) as well as in white blood cells of cancer patients (Malayappan et al., 2010). PM-induced cell death was observed in our SIGC cells after 48 h. We found that PM binds to DNA to first form a NOR-G-OH (m.wt: 239) adduct followed rapidly by formation of the G-NOR-G (m.wt: 372) adduct. These data
support that DNA adducts formation is involved in the ovotoxic, in addition to the antineoplastic properties of PM.

ATM is a protein kinase that is recruited and activated by DNA DSBs. Various chemicals including CPA activate ATM signaling eventually resulting in apoptosis in mammalian cells (Ganesan et al., 2013; Toulany et al., 2014). ATM phosphorylates histone H2AX in response to DNA DSBs (Burma et al., 2001) and γH2AX protein was previously localized within oocyte and granulosa cell of PM exposed mouse ovaries (Petrillo et al., 2011). Both ATM activation and γH2AX appearance serve as proxy markers of cellular DNA damage (Tanaka et al., 2006). Increased Atm mRNA and protein were observed after 24 h of exposure to 6 µM PM, and by both PM concentrations after 48 h, indicating a dose- and time-dependent impact of PM on Atm induction. We report herein that PM increased the percentage of γH2AX-positive cells containing and also increased γH2AX protein in total ovarian protein homogenates in both a dose- and time-dependent manner. These results indicate that DNA damage is induced by PM exposure after DNA adducts formation in rat ovarian granulosa cells.

Poly(ADP-ribose) polymerase-1 (PARP-1) is rapidly activated by DNA DSBs, and involved in the posttranslational modification of nuclear proteins during DNA damage and activates cellular process like DNA replication, DNA repair, apoptosis and genome stability (Yu et al., 2002). In the current study, Parp1 mRNA and protein were increased in a similar manner to that observed in Atm - by 6 µM PM after 24 h of PM exposure and by both PM concentrations after 48 h. A link between ATM and PARP-1 has been previously found; PARP-1 deficient mice were extremely sensitive to low doses of γ-radiation, and this phenotype could be ascribed to a deficient ATM-kinase activation in tissues such as the
intestine epithelium (de Murcia et al., 1997). In our current study, ATM protein level was coincident with PARP-1 protein level after PM exposure.

PARP-1 competes with XRCC6 to repair a DSB as part of the NHEJ pathway but, in contrast, PARP-1 is also recruited to a DSB in the absence of XRCC6 when the classical pathway of NHEJ is absent (Wang et al., 2006). XRCC6 is a KU protein which forms a heterodimer with XRCC5. Deficiency of XRCC6 protein causes genomic instability and malignant formation (Wang et al., 2013). We found that Xrcc6 mRNA was reduced by the lower PM exposure after 24 h, but increased by the 6 µM PM concentration at the same time point. Subsequently, Xrcc6 mRNA in 3 µM PM-treated cells returned to control levels, while it was reduced at the higher PM concentration. XRCC6 protein levels were initially increased by the 6 µM PM exposure, but subsequently decreased. These results indicate that there is a dynamic response of Xrcc6 mRNA in response to PM exposure, and may suggest that XRCC6 is involved in the earlier DDR via the NHEJ repair pathway, but at later times of exposure, that PARP-1 could potentially compete with XRCC6 to repair DNA damage through an XRCC6-independent DNA repair pathway, however, these possibilities cannot be confirmed by the current study.

PRKDC is a DNA-dependent protein kinase recruited and activated at the site of DNA damage by KU proteins to repair DSBs repair by NHEJ pathways. Interestingly, capsaicin-induced autophagy of MCF-7 cells was shown to occur via activation of the PRKDC, ATM and PARP-1 DDR proteins (Yoon et al., 2012). Further, increased ionizing radiation-induced Prkdc mRNA was observed in mouse leukaemic monocyte macrophage cell line RAW264.7 and was thought to be involved in prevention of cell death (Dhariwala et al., 2012). We found a dose-dependent temporal pattern of Prkdc mRNA and protein increased expression
in response to PM exposure in SIGC cells. Our study also indicates that PM could induce PRKDC as part of the mechanism to repair DNA damage and improve the survivability of SIGC cells by preventing cell death.

BRCA1 is breast cancer protein required for repair of DSBs via the HR pathway. Previously, we have found that Brca1 mRNA and protein was increased in response to a chemical exposure (7,12-dimethylbenz[a]anthracene) that induced DSBs in rat ovaries (Ganesan et al., 2013). Activation of ATM and BRCA1 inhibited apoptosis induced by 5-azacytidine by repairing DNA damage in cells that were resistant to 5-azacytidine and this activation was absent in the parental 5-azacytidine-sensitive leukemia cell line U937 and HL-60 (Imanishi et al., 2014). In this study, we observed a temporal pattern of increased Brca1 mRNA and protein due to PM exposure, supporting that the HR DDR pathway is induced and that ovarian Brca1 is responsive to PM exposure.

BRCA1 can interact with RAD 51 during HR to regulate the function of RAD51 in response to DSBs and block sister chromatid replication slippage (Cousineau et al., 2005). Down regulation of RAD51 previously indicated failure of the HR pathway in hypoxic cancer cells (Bindra et al., 2004). Also, Rad51 mRNA was elevated in tumor cells, suggesting that it plays a role in repair of DNA damage (Raderschall et al., 2002). We found that PM increased Rad51 mRNA and protein at the 6 µM concentration and that a similar response occurred with the 3 µM PM exposure, but this lagged that of the higher concentration, again indicating a dynamic, temporal pattern of the DDR to PM exposure.

In conclusion, PM exposure to an SIGC line caused formation of both NOR-G-OH and G-NOR-G DNA adducts. In addition, loss of cell viability and induction of the DDR, in a time- and dose-dependent manner was observed. Taken together, PM, the active metabolite
of CPA, may induce ovarian cell death via formation of DNA adducts and the ovarian granulosa cells induce mechanisms to respond to this genotoxic insult likely in order to protect the ovarian granulosa cells.

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**References**


### Table 1. Primer sequences used for qRT-PCR

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<th>Reverse primer</th>
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Figure 1. Formation of Phosphoramide mustard-induced DNA adducts

PM undergoes nonenzymatic degradation to produce nornitrogen mustard (NOR). Both PM and NOR alkylate guanine bases within DNA to form guanine monoadducts, N-[2-(N7-guaninyl)ethyl]-N-[2-hydroxyethyl]-amine (G-NOR-OH), and DNA-DNA cross-links, N,N-bis[2-(N7-guaninyl)ethyl] amine (GNOR-G), although PM is considered the major DNA reactive metabolite (Malayappan et al., 2010).
Figure 2. PM decreases % of cell viability

SIGC cells were treated with DMSO or PM (0.5, 1, 3 or 6 µM) for 48h. Trypan blue staining was used to determine the cell viability. Values represent % of viable cells ± SE. * = P < 0.05; different from control.
Figure 3. PM exposure induces DNA adduct formation

SIGC cells were treated with DMSO or PM (3 or 6 µM) for 12, 24 or 48h for DNA isolation. Agilent QTOF6540 LC/MS analysis was performed to determine the DNA adducts formation. The (A,B) NOR-G-OH was observed after 24h of exposure to 6 µM PM, while (C,D) G-NOR-G was formed after 48h at both the 3 and 6 µM concentrations. Values represent the relative abundance of DNA adduct /10^5 nucleotides).
Figure 4. PM induces DNA damage formation

SIGC cells were treated with DMSO or PM (3 or 6 µM) for 24 or 48h and γH2AX protein localized using immunofluorescence (A-C). Blue staining represents DNA; Red staining indicates γH2AX. (D) Values represent % of γH2AX positive cells ± SE. Different letters difference from CT; P < 0.05.
Figure 5. PM exposure increases DDR gene mRNA expression levels

RNA was isolated from SIGC cells treated with DMSO or PM (3 or 6 µM) for (A) 24 h or (B) 48 h, followed by qRT-PCR. Genes were normalized with Gapdh and values reported as fold change relative to a control value of 1 ± SEM. Different letters difference from CT; $P < 0.05$. 

A

B
Figure 6. DDR proteins are increased in response to PM exposure

Total protein was isolated from SIGC cells treated with DMSO or PM (3 or 6 µM) for (A) 24 h or (B) 48 h, followed by Western blotting. Ponceau S was used for normalization with protein of interest and values reported as raw mean value ± SEM. Different letters differ from CT; $P < 0.05$. 
CHAPTER 7. THE OVARIAN DNA DAMAGE REPAIR RESPONSE IS INCREASED PRIOR TO PHOSPHORAMIDE MUSTARD-INDUCED FOLLICLE DEPLETION, WHICH IS PREVENTABLE BY INHIBITION OF ATAXIA TELANGIECTASIA MUTATED

A paper formatted for submission to Toxicological sciences

Shanthi Ganesan and Aileen F. Keating

Contribution Statement:

I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Aileen F. Keating designed experiments, aided in data interpretation and edited the manuscript.

Abstract

Phosphoramide mustard (PM) is an ovotoxic metabolite of cyclophosphamide and destroys primordial and primary follicles potentially by DNA damage induction. The temporal pattern by which PM induces DNA damage and initiation of the ovarian DNA damage response has not yet been characterized. This study investigated DNA damage initiation, the DNA repair response, as well as induction of follicular demise using a neonatal rat ovarian culture system. Additionally, to delineate specific mechanisms involved in the ovarian response to PM exposure, utility was made of PKC delta (PKCδ) deficient mice as well as an ATM inhibitor (KU-55933; AI). Fisher 344 PND4 rat ovaries were cultured for 12, 24, 48 or 96 h in medium containing DMSO ± 60 µM PM or KU-55933 (10 nM). PKCδ WT and KO mice were cultured with DMSO ± 10 µM PM for 144 h. PM-induced activation of DNA damage repair genes was observed as early as 12 h post-exposure. The abundance of ATM, PARP1, E2F1, P73 and CASP3 were increased but RAD51 and BCL2 protein decreased after 96 h of PM exposure. PKCδ deficiency reduced numbers of all follicular stages, but did not have an additive impact on PM-induced ovotoxicity. ATM inhibition
protected all follicle stages from PM-induced depletion in number. In conclusion, the induction of ovarian DNA damage repair response post-PM exposure, suggest that DNA damage is involved in PM-induced ovotoxicity.

**Introduction**

The female gamete, the oocyte, is produced by the ovary, wherein they become integrated into follicular structures. Females are born with a finite number of oocytes, which once depleted, cannot be replaced (Hirshfield et al., 1991). Approximately 10% of women suffer infertility due to premature depletion of their oocyte pool (Nikolaou and Templeton, 2003). There are a number of environmental exposures, including cigarette smoke, occupational chemicals, pesticides and chemotherapeutic agents that can destroy oocytes, potentially hastening entry into premature ovarian senescence (Hoyer and Sipes, 1996). Those chemotherapeutic chemicals that are alkylating or DNA damaging agents reduce follicle number and fertility with concomitant induction of amenorrhea (Hudson, 2010). Moreover, chemotherapeutic agents may potentially have adverse effects on the offspring of the treated individual; that is, early embryonic mortality resulting from genetic effects, such as chromosomal aberrations that are induced in exposed oocytes (Gonfloni et al., 2009).

Cyclophosphamide ( CPA) is used to treat cancer and autoimmune diseases, and causes premature ovarian failure and rapid amenorrhea in treated individuals by follicular destruction (Hudson, 2010). In rodents, CPA targets primordial follicles in mice (Plowchalk and Mattison, 1992) and antral follicles in rats (Ataya et al., 1990). Phosphoramide Mustard (PM), an ovotoxic metabolite of CPA, destroys rapidly dividing cells by covalently binding to DNA, inducing DNA-DNA, DNA-protein cross links and DNA double strand breaks
PM caused dose- and time-dependent primordial and primary ovarian follicle loss following DNA damage in mice and rat oocytes (Petrillo et al., 2011). If DNA repair fails, or the cell is overwhelmed by too many DNA lesions, cellular sensors initiate apoptosis (Friedberg, 2003). The main players of DNA damage recognition are ataxia telangiectasia mutated (ATM), ATM related (ATR) and DNA-dependent protein kinases (DNA-PKcs), which phosphorylate a multitude of proteins and thereby induce the DNA damage response (DDR), in which P53 and BRCA1/2 play important roles (Roos and Kaina, 2006). ATM is mainly activated by DSBs formed either directly by ionizing radiation (IR), or indirectly by methylating agents or cisplatin exposure (Caporali et al., 2004). ATM is implicated in three crucial functions: regulation and stimulation of DSB repair, activation of cell cycle checkpoints, and signaling for apoptosis induction; three key nodes in making the decision between cellular survival and death following genotoxin exposure (Roos and Kaina, 2012).

Protein kinase c (PKC) is a serine-threonine kinase that is activated by diverse stimuli and which participates in cellular processes such as growth, differentiation and apoptosis (Hug and Sarre, 1993). PKC delta (PKCδ) represents a subfamily of novel PKC isoforms, is widely expressed in the rat ovary and regulated by estrogen (Peters et al., 2000). The catalytic fragment of PKCδ interacts with P73β and phosphorylates the transactivation and DNA binding domains of P73β to induce its apoptotic function (Ren et al., 2002). However, PKCδ also enhances proliferation and survival of murine mammary cells (Grossoni et al., 2007), thus indicating that PKCδ has both pro- and anti-apoptotic properties.

DNA damage can activate the extrinsic death receptor apoptosis pathway (Fas) and/or the intrinsic mitochondrial apoptosis pathway (Roos and Kaina, 2006). In the mitochondrial
apoptotic pathway, voltage-dependent anion channel (VDAC) regulates the release of apoptotic proteins, such as cytochrome c (CYCS), from mitochondria by oligomerization (Igosheva et al., 2010). CYCS release is also regulated by the BCL-2 family protein BAX, which interacts with VDAC to increase pore size and promote CYCS release, while the anti-apoptotic protein BCL-XL has the opposite effect (Wu et al., 2010).

Although it is recognized that infertility ensues from PM treatment, little is known about the earliest mechanistic events that contribute to ovotoxicity or that could provide protection from PM exposure. Our previous evidence using spontaneously immortalized granulosa cells indicated that PM exposure results in DNA adduct formation, followed by increased expression of members of the DNA repair response, thus we hypothesized that ATM and PKCδ could be important cellular sensors of ovarian DNA damage. This study was designed to investigate the temporal pattern of ovarian cellular signaling events post-PM exposure, using a whole neonatal ovarian culture method, with inclusion of an ATM inhibitor and use of a mouse strain that was deficient in PKCδ.

Methods and Materials

Reagents

Phosphoramide Mustard (National Cancer Institute, Bethesda, MD), Bovine serum albumin (BSA), Ascorbic acid, Transferrin, 2- β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, Ammonium persulphate, Glycerol, N'N'N'-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, Sodium chloride and KU-55933, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham's F12), Albumax, Penicillin (5000U/ml), Hanks'
Balanced Salt Solution (without CaCl2, MgCl2 or MgSO4) from Invitrogen Co. (Carlsbad, CA). Millicell-CM filter inserts and 48 well cell culture plates were obtained from Millipore (Bedford, MA) and Corning Inc. (Corning, NY) respectively. RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, RT<sup>2</sup> First Strand Kit, RT<sup>2</sup> SYBR Green Mastermix, Quantitect<sup>TM</sup> SYBR Green PCR kit and DNA damage RT<sup>2</sup> profiler PCR array kit for rats were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from Iowa State University DNA facilities. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). The polyclonal goat anti-rabbit secondary were obtained from Pierce Biotechnology (Rockford, IL). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

**Ovary culture**

Ovaries were collected from PND4 female F344 rats or PKCδ wild type and deficient mice and cultured as described by Devine, *et al.*, 2002. Briefly, PND4 female pups were euthanized, ovaries removed, trimmed of excess tissue and placed onto a membrane floating on 250 µl of DMEM/Ham's F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 µg/ml ascorbic acid, 5 U/ml penicillin and 27.5 µg/ml transferrin per well in a 48 well plate previously equilibrated to 37 °C. Ovaries were cultured in medium containing vehicle control (DMSO) +/- PM 60 or 10 µM) and maintained at 37 °C with 5% CO2 for 12, 24, 48, 96 or 144 h and medium replaced every two days. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries exposed to PM treatments (60 µM) were cultured in a separate incubator from other treatments to eliminate contamination from an ovotoxic, PM-generated volatile metabolite (Madden *et al.*, 2014).
Ovarian follicle counts

PND4 female F344 rats ovaries were cultured in medium containing vehicle control (DMSO) ± PM (60 µM) ± KU-55933 (10 nM) and maintained at 37 °C with 5% CO2 for 96 h. The ATM inhibitor KU-55933 concentration was chosen based on our observations that primordial follicular viability was unaffected (data not presented). Ovaries from PKCδ wild type and deficient mice (Gift from Dr. Kanthasamy, Iowa State University) were cultured in vehicle control (DMSO) ± PM (10 µM) for 144 h. The chosen PM concentration of 10 µM was previously characterized to cause follicle depletion from day 2 onward after a single exposure in CD-1 mouse ovaries (Desmeules and Devine, 2006). Following incubation, ovaries were placed in 4% paraformaldehyde for 2 h and transferred to 70% ethanol. Ovaries were embedded in paraffin following standard histological procedures. Tissue sections (5 µM) were cut and stained with haematoxylin and eosin and every 6th section were mounted as described previously (Igawa et al., 2009). Healthy follicles were counted (n=5 / group) and follicle population was classified according to the procedure adopted from (Devine et al., 2004).

RNA isolation and RT² profiler PCR Array

RNA was isolated (n = 3; 10 ovaries per pool) using an RNeasy Mini kit and the concentration determined using a NanoDrop (λ = 260/280 nm; ND 1000; Nanodrop technologies Inc., Wilmington, DE). Three biological replicates were used to perform a RT² profiler DNA damage PCR array. The PCR array contained 96-wells, each containing a gene-specific primer set; therefore one plate tested 96 genes per biological sample. Total ovarian RNA (1 µg) was reverse transcribed to cDNA using an RT² first-strand Kit, combined with an appropriate RT² SYBR Green Mastermix before being aliquoted into the
wells of the RT² Profiler PCR Array. The regular cycling program consisted of a 10 min hold at 95°C and 40 cycles of denaturing at 95°C for 15 s and a combined annealing and extension for 1 min at 60°C. Each gene was normalized to a housekeeping genes on the array; ribosomal protein, large, P1 (Rplp1), as recommended by the company-provided analysis software. There was no effect of PM exposure on ovarian mRNA levels of this housekeeping gene. The SA Biosciences RT² Profiler PCR Array Data Analysis software quantified the changes in mRNA levels using the $2^{-\Delta\Delta C_t}$ method.

**qRT-PCR**

Total RNA (1 μg) was reverse transcribed to cDNA utilizing the Superscript III One-Step qRT-PCR kit. cDNA was diluted (1:20) in RNase-free water and amplified on an Eppendorf PCR Master cycler using a Quantitect SYBR Green PCR kit. Primers for Atm, Prkdc, Xrcc6, Parp1, Rad51, Brca1 and Gapdh were designed by Primer 3 Input Version (0.4.0) (Ganesan and Keating, 2014). The regular cycling program consisted of a 15-min hold at 95°C and 45 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 20 s at which point data were acquired. Each sample was normalized to Gapdh before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

**Protein isolation and Western blotting**

Total protein was isolated from cultured ovaries (n=3; 10 ovaries per pool via homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Ganesan et al., 2013). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a BCA protocol. Protein was stored at -80°C until
use. SDS-PAGE was used to separate proteins which were then transferred to nitrocellulose membrane. Membranes were blocked for 1 h in 5% milk in Tris-buffered saline containing tween 20, followed by incubation with anti-PARP1 antibody (1:200), anti-ATM antibody (1:100), anti-RAD 51 antibody (1:500), anti-E2F1 antibody (1:100), anti-P73 antibody (1:200), anti-BCl2 antibody (1:200) and anti-Caspase 3 antibody (1:50) overnight at 4°C. Following three washes in TTBS (1X) membranes were incubated with an appropriate species-specific secondary antibody (1:2000) for 1 h at room temperature. Membranes were washed three times in TTBS, once in TBS and then incubated in enhanced chemiluminescence detection substrate for 5 min and exposed to X-ray film. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). All proteins were normalized to Ponceau S densitometry values.

**Statistical analysis**

Treatment comparisons for follicle count experiments were performed using one-way analysis of variance. Quantitative RT-PCR and Western blot data were analyzed by t-test comparing treatment with control raw data at each individual time point. RT² Profiler PCR array data were analyzed using the online SA Biosciences software which quantified the changes in mRNA levels using the $2^{-\Delta \Delta C_{T}}$ method. All other statistical analysis was performed using Prism 5.04 software (GraphPad Software). Statistical significance was defined as $P < 0.05$, with a trend for a difference considered at $P < 0.1$. For graphical purposes, protein expression is presented as a mean ± SE, relative to the respective control.
Results

Impact of PM exposure on abundance of genes encoding proteins involved in the ovarian response to DNA damage

After 24 h of PM (60 µM) exposure, of the 89 genes tested, fourteen genes had increased \((P < 0.05)\) gene expression, and four genes were trending toward being increased \((P < 0.10)\) compared with the controls. After 48 h of PM exposure, of the 89 genes tested, five genes had increased \((P < 0.05)\) gene expression, and six genes were trending toward being increased \((P < 0.10)\) and two genes were trending toward being decreased \((P < 0.10)\) compared to control-treated ovaries (Table 1).

In addition to genes present on the PCR array, primers were designed to quantify changes in genes that were of interest and were not either present on the array or the array was not performed at the time point of interest. Following 12 h of PM exposure, \textit{Atm} (3.4-fold ± 0.7); \textit{Parp1} (1.6-fold ± 0.5); \textit{Prkdc} (0.9-fold ± 0.3); \textit{Xrcc6} (1.4-fold ± 0.3); \textit{Rad51} (1.9-fold ± 0.5) and \textit{Brcal} (1.9-fold ± 0.6) mRNA abundance were increased \((P < 0.05)\) compared to control (Figure 1).

Following 24 h of PM exposure, \textit{E2f1} (1.65-fold ±0.15) and \textit{p73} (3.06-fold ± 0.7) mRNA levels were increased \((P < 0.05)\) compared to control. In contrast, PM exposure decreased \((P < 0.05)\) \textit{Vdca1} (0.4-fold ± 0.1) mRNA abundance relative to control. There was no impact of PM exposure on \textit{Cycs} mRNA level (Figure 2A). After 48 h of PM exposure, \textit{E2f1} (1.4-fold ± 0.2); \textit{p73} (90-fold ± 1); \textit{Mdm2} (0.7-fold ± 0.2); \textit{Vdca1} (0.4-fold ± 0.2) and \textit{Cycs} (2.5-fold ± 0.5) mRNA levels were increased \((P < 0.05)\) compared to control-treated ovaries (Figure 2B). After 96 h of PM exposure, increased \((P < 0.05)\) levels of \textit{E2f1} (1.3-fold ± 0.2); \textit{p73} (1.4-fold ± 0.5); \textit{Mdm2} (1-fold ± 0.07); \textit{Vdca1} (0.3-fold ± 0.06) and \textit{Cycs} (0.7-fold ± 0.1) mRNA were observed compared to control (Figure 2C).
Effect of exposure to PM on abundance of DNA damage response proteins

The DNA damage repair proteins ATM (PM: 1.6 ± 0.3; CT: 1.4 ± 0.02) and PARP1 (PM: 1.8 ± 0.03; CT: 1.5 ± 0.02) were increased ($P < 0.05$) compared to ovaries cultured in control media. In contrast, RAD51 (PM: 1.4 ± 0.02; CT: 1.5 ± 0.02) protein abundance was decreased ($P < 0.05$) after PM exposure (Figure 3A). The transcription factors E2F1 (PM: 1.5 ± 0.06; CT: 0.8 ± 0.04); P73 (PM: 1.9 ± 0.1; CT: 1.3 ± 0.03) (Figure 3B) and apoptotic proteins CASP3 (PM: 1.5 ± 0.02; CT: 1.3 ± 0.01) (Figure 3C) were increased ($P < 0.05$) after PM exposure, relative to control-treated ovaries. In contrast, the anti-apoptotic protein BCL2 (PM: 0.9 ± 0.05; CT: 1.2 ± 0.02) was decreased ($P < 0.05$) after PM exposure compared to control ovaries (Figure 3D).

Involvement of ATM protein in ovotoxicity induced by PM exposure

Primordial follicle number (PM: 279.7 ± 7.8; CT: 568 ± 26.2; AI: 578 ± 49.1; PM+AI: 536 ± 9.5) were reduced ($P < 0.05$) after PM treatment. There was no impact of ATM inhibition on primordial follicle number. Interestingly, in the presence of ATM inhibition, primordial follicles were protected from PM-induced depletion (Figure 4A).

PM exposure reduced ($P < 0.05$) small primary follicle number (PM: 40.3 ± 8; CT: 128.7 ± 5.8; AI: 124 ± 4.7; PM + AI: 69 ± 7.2), compared to all other groups. There was no impact of ATM inhibition on small primary follicle number, however, ATM inhibition partially prevented ($P < 0.05$) PM-induced destruction of small primary follicles (PM + AI: 69 ± 7.2; PM: 40.3 ± 8) (Figure 4B).

A similar pattern was observed for both large primary and secondary follicles. PM exposure depleted ($P < 0.05$) large primary follicle number (PM: 13 ± 3.2; CT: 32.6 ± 2.3; AI: 51.6 ± 2.9; PM + AI: 32.3 ± 4.4). Interestingly, ATM inhibition increased large primary
follicle numbers (AI: 51.6 ± 2.9; PM: 13 ± 3.2; CT: 32.6 ± 2.3; PM + AI: 32.3 ± 4.4), compared to all other groups (Figure 4C), and ATM inhibition prevented PM-induced depletion of large primary follicles (PM + AI: 32.3 ± 4.4; PM: 13 ± 3.2).

Secondary follicle numbers were reduced ($P < 0.05$) by PM exposure (PM: 2.6 ± 1.4; CT: 20.6 ± 3.8; AI: 18.6 ± 2.1). ATM inhibition alone did not impact secondary follicle number (CT: 20.6 ± 3.8; AI: 18.6 ± 2.1), and ATM inhibition partially protected secondary follicles from PM-induced ovotoxicity (PM: 2.6 ± 1.4; PM+ AI 12.3 ± 1.7) (Figure 4D).

**Impact of PKCδ deficiency on PM-induced ovotoxicity**

PKC delta (PKCδ) wild type (WT) and deficient (KO) mouse ovaries were cultured with vehicle control (DMSO) ± PM (10 µM) for 144 h. PKCδ deficiency (KO) alone reduced ($P < 0.05$) ovarian follicle number, relative to the WT mice; primordial (WT: 193 ± 39.4; KO: 84 ± 22.3), small primary (WT: 59.2 ± 14.2; KO: 24 ± 3.9), large primary (WT: 28 ± 4.6; KO: 18.4 ± 5.1) and secondary (WT: 6.6 ± 1.4; KO: 0.4 ± 0.4) follicles were all lower than those contained in ovaries from WT mice. PM reduced number of healthy follicles in both WT and KO mice ovaries (Figure 5A). The percentage of PM-induced follicle loss was calculated for both genotypes and no impact of PKCδ deficiency on PM-induced follicle loss was observed (Figure 5B).

**Discussion**

Cells continuously experience multiple types of DNA modifications that occur due to external sources or exposures and DNA is the main victim of environmental genotoxins including alkylating compounds, ultraviolet (UV) light and ionizing radiation. PM, an alkylating agent causes both dose- and time-dependent ovarian primordial and primary
follicle loss following DNA damage to mice and rat oocytes (Petrillo et al., 2011). No mechanistic studies have been performed on the ovarian response to PM-induced DNA damage thus; we explored a potential mechanism of PM-induced ovarian follicle loss in neonatal cultured rat ovaries. Using this system, we previously demonstrated that PM causes depletion of all follicle types from day 4 onwards (Madden et al., 2014).

Two approaches were employed to determine transcriptional effects of PM exposure on genes involved in the ovarian response to DNA damage. We first used a commercially available PCR array to survey a wide number of genes at either 24 or 48 h post-exposure. A dynamic response in terms of gene activation was observed, in that genes that were altered after 24 h included Atm, Bax, Bbc3, Cdkn1a, Ddit3, Ercc2, Exo1, Fancc, Gadd45g, Lig1, Mgmt, Nthl1, Pold3, Rad21, Rpa1, Topbp1, Ung and Xrcc2. After 48 h of exposure, genes that remained elevated included Atm, Bbc3, Cdkn1a, Nthl1, Mgmt and Pold3, however, a number of genes returned to control levels and genes that were increased at 48 h but not 24 h were Bard1, Gadd45a, Mif, Mpg, Rad18 and Terf1. We chose to also examine a number of genes after 12 h of PM exposure by traditional qPCR and found that Atm, Parp1, Prkdc, Xrcc6, Rad51 and Brca1 were elevated in mRNA abundance, and with the exception of Atm, all were returned to control level by the 24 h time point. Thus, a temporal ovarian response to PM exposure was evident and increased PM-induced Atm was sustained. Activation of these genes support that DNA DSBs were induced by PM exposure as seen in other experimental paradigms (Durocher and Jackson, 2001; Ganesan and Keating, Under Review) and indicate that PM-induced DNA damage was occurring prior to activation of the ovarian DNA repair mechanism. These data also support that PM induces DNA damage as an upstream, potentially initiating event of PM-induced follicle loss and that the ovary activates DNA
repair genes to protect against PM-induced ovotoxicity. DNA damage repair response protein abundance was quantified at the time of follicle loss. ATM and PARP1 were increased while RAD51 was decreased by PM exposure. Since ATM, PARP1 and RAD51 are all involved in the DSB repair pathway, the decreases in RAD51 was unexpected, however, the increase in ATM and PARP1 further support that the ovary is mounting a protective response to PM exposure that has caused an assault on the germline DNA.

Upon phosphorylation of P53 by ATM, P53 becomes stabilized and blocks cell proliferation by up-regulation of P21 (CDKN1a), which triggers G1-S arrest (Roos and Kaina, 2006). In this study, Cdkn1a gene was increased after 24 h of PM exposure but there were no alterations in p53 mRNA abundance. Low levels of P53 during DSBs are sufficient to propel the transcription of the Cdkn1a gene to result in cell-cycle arrest (Roos and Kaina, 2006). In addition, both ATM and ATR phosphorylate CHK1 and CHK2 to activate the transcriptional regulator E2F1 in response to DNA damage and E2F1 then can induce apoptosis through a P53-independent pathway (Phillips et al., 1997). In human cancer cell lines, E2F1 was activated by etoposide which in turn stimulated the transcription of the p73 gene in human cancer cell lines (Urist et al., 2004). In this study, both E2f1 and p73 mRNA levels were increased after 24 h and the protein elevated after 96 h of PM exposure, potentially indicating that PM induces apoptosis independent of P53. MDM2 degrades P53 using a negative feedback loop resulting in a decrease in exogenous P53 in transient transfection experiments or in endogenous P53 induced by DNA damage (Wang et al., 2001). The transcriptional level of Mdm2 was increased after 24 h while there were no changes in p53 mRNA expression which might be due to an increased level of P73.
Transcriptional up regulation of *Bbc3* (PUMA) resulted in increased BAX mitochondrial translocation and CYCS release during P73-induced apoptosis (Melino *et al.*, 2004). PM increased *Bbc3* and *Bax* gene expressions after 24 h of exposure, but decreased BCL2 protein level after 96 h. This observation was similar to another study in which docetaxel/doxorubicin chemotherapy decreased BCL2 in human breast cancer cells (Buchholz *et al.*, 2003). BCL-2 inhibits apoptosis by preventing the release of mitochondrial apoptogenic factors such as CYCS and apoptosis-inducing factor (AIF) into the cytoplasm. This study indicates that PM activates *Bbc3* and *Bax* and decreases BCL2 to cause mitochondrial apoptotic cell death.

VDAC1 is another key player in mitochondria-mediated apoptosis, participating in the release of mitochondrial pro-apoptotic CYCS to the cytosol (Zaid *et al.*, 2005). We found that PM increased *Vdac1* and *Cycs* gene expressions after 24 h in rat ovaries. These data are similar to a study that demonstrated increased *Vdac1* expression after CPA exposure which stimulated apoptosis in ovarian granulosa cells (Zhao *et al.*, 2010). It is recognized that CYCS release activates both CASP9 and formation of apoptosome to cause apoptosis (Ishida, 2004). In this study, CASP3 protein was increased after 96 h by PM exposure, the time point at which PM-induced ovotoxicity is occurring (Madden *et al.*, 2014). Increased CASP3 has also been demonstrated during apoptosis induced by another ovotoxicant, 7,12-dimethylbenz[a]anthracene (Ganesan *et al.*, 2013).

Since *Atm* mRNA expression was increased at the earliest time point examined (12 h) and was sustained at an elevated level over the time course of PM exposure, we investigated a functional role for ATM during PM exposure using the ATM inhibitor KU-55933. Interestingly, though no apparent impact of ATM inhibition on primordial, small primary or
secondary follicles was observed, lack of ATM increased the number of large primary follicles, potentially indicating a role for ATM in viability and/or maturation of this follicular subtype. Lack of ATM protected all follicle types from the ovotoxic impacts of PM. This result was similar to a previous study that showed that ATM inhibitor improved oocyte survival after doxorubicin exposure by activation of c-AbI-Tap63 pathway (Soleimani et al., 2011). It has been shown previously that blocking ATM activity caused induction of ATM protein, which is regulated by p73 and E2f1 transcriptional level (Khalil et al., 2012). These results are in correlation with our findings in the ovary, and suggest that ATM serves to eliminate cells with excessive DNA damage (Herzog et al., 1998). Whether the follicles in the ovarian treated with PM plus ATM inhibitor are “healthy” is doubtful and thus protection from PM-induced ovotoxicity may not necessarily result in benefits for fertility.

PKCδ is a member of the novel PKC subfamily and catalytic fragment of PKCδ-mediated phosphorylation of P73β is associated with accumulation of P73β and induction of P73β-mediated transactivation (Ren et al., 2002). Since P73 is increased by PM, we utilized available PKCδ wild type and deficient mice to determine the role of PKCδ in ovarian folliculogenesis and any involvement in PM-induced ovotoxicity. PKCδ knockout reduced the numbers of all stage follicles compared to wild type ovaries. When these mice were exposed to PM, the number of healthy follicles was reduced by PM in both the wild type and deficient mice. However, there was no additional impact of PM exposure on follicle deletion percentage due to lack of PKCδ. PKCδ enhanced proliferation and survival of murine mammary cells (Grossoni et al., 2007) indicating it’s involvement in cell viability in extra-ovarian tissues. These results indicate that PKCδ is important for ovarian follicle survival.
In summary, the ovary induces increased levels of mRNA and proteins involved in the response to DNA damage following PM exposure, supporting that induction of DNA damage is an initiating event of PM-induced ovotoxicity. However, the repair mechanisms were reduced and cell death mechanisms were increased at the time of follicle loss. PM-induced apoptosis appears P53-independent but is potentially occurring through an E2F1-P73 dependent mitochondrial apoptotic pathway. In addition, the data support that inhibition of ATM prevented elimination of PM-affected oocytes, which may not necessarily represent a benefit to the germline. PKCδ is involved in either ovarian endowment or follicular survival but there was no impact of PKCδ deficiency on PM-induced ovotoxicity. The data shed additional light on potential targets for amelioration of PM-induced infertility that occurs as a side effect to anti-neoplastic therapy.

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References


Table 1. PM exposure alters DNA damage response genes

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Fisher 344 PND4 rat ovaries were cultured for 24 or 48 h in medium containing vehicle control ± 60 µM PM. RNA was isolated and used to perform a RT² Profiler PCR array. Values represent fold-change ± SEM relative to a control value of 1 (10 ovaries per pool), normalized to Rplp1. * = different from control, $P < 0.05$; † = $P < 0.1$. 

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Figure 1. PM activates DNA repair genes prior to follicle loss

Fisher 344 PND4 rat ovaries were cultured for 12 h in medium containing vehicle control ± 60 µM PM. RNA was isolated and used to perform qRT-PCR. Values are expressed as mean fold change relative to control treated ovaries ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05.
Figure 2. PM activates transcription factor and apoptotic gene mRNA level

Fisher 344 PND4 rat ovaries were cultured for (A) 24, (B) 48 or (C) 96 h, in medium containing vehicle control ± 60 µM PM. RNA was isolated and used to perform qRT-PCR. Values are expressed as mean fold change relative to control treated ovaries ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = $P < 0.05$. 
Figure 3. PM activates E2F1-P73 dependent apoptotic pathway

Following 96 h of PM exposure, protein was isolated and used to perform western blot analysis for (A) DNA damage response proteins, (B) Transcription factor proteins, (C) pro-apoptotic CASP3 and (D) anti-apoptotic BCL-2. Results were normalized to Ponceau S densitometric staining and expressed as mean raw data ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = $P < 0.05$. 
Figure 4. ATM inhibition prevents PM-induced ovotoxicity

Ovaries were cultured in medium containing vehicle control (DMSO) ± PM (60 µM) ± KU-55933 (10 nM) for 96 h. Following culture, follicles were classified and counted. Total number of primordial (A), small primary (B), large primary (C) and secondary (D) follicles are presented as mean raw data ± SE; n=5. Different letters indicates statistical difference from control treated ovaries at $P < 0.05$. (E) Histological ovarian sections of the control treated (CT), PM treated (PM), ATM inhibitor treated (AI) and PM plus ATM inhibitor treated (PM + AI) are presented.
Figure 5. PKCδ is essential for follicular survival

Ovaries from PKCδ wild type (WT) or knockout (KO) mice were cultured with vehicle control (DMSO) ± PM (10 µM) for 144 h. Following culture, follicles were counted between (A) treatments and strains and (B) the percentage reduction in follicle stage number calculated. The total number of primordial, small primary, large primary and secondary follicles are expressed as mean raw data ± SE; n=5. Statistical significance was defined as * = P < 0.05 between treatments, while differences between strains within each follicle group are designated with letters. (C) Histological ovarian sections of the wild type control treated (WT CT), wild type PM treated (WT PM), knockout control treated (KO CT) and knockout PM treated (KO PM) are presented.
CHAPTER 8. OBESITY ALTERS THE PHOSPHORAMIDE MUSTARD-INDUCED DNA REPAIR RESPONSE IN MOUSE OVARIES

A paper formatted for submission to Biology of Reproduction

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Contribution Statement:

I performed all analyses on ovarian tissue in this paper, designed the experiments, interpreted data and wrote the paper. Jackson Nteeba aided with designing experiment and tissue collection. Jill Madden aided in tissue collection. Aileen F. Keating designed the experiments, aided in data interpretation and edited the manuscript.

Abstract

Phosphoramide mustard (PM), an ovotoxic metabolite of cyclophosphamide, destroys rapidly dividing cells at least partly, by inducing DNA damage. γH2AX, marker of DNA damage was evident in ovaries from obese mice indicated that obesity increased sensitivity to another ovotoxicant, 7,12-dimethylbenz(a)anthracene. This study investigated the effects of PM exposure on DNA damage induction and activation of DNA damage repair in both lean and obese female mice. Wild type (lean) non agouti (a/a) and KK.Cg-Ay/J heterozygote (obese) mice were dosed once with sesame oil or PM (95%; 25 mg/kg; i.p). Obesity increased ($P < 0.05$) liver while decreased ($P < 0.05$) uterine and spleen weights regardless of body composition, however, decreased ($P < 0.05$) ovarian weight was only observed in the obese females exposed to PM. Exposure to PM decreased ($P < 0.05$) primordial and primary ($P < 0.05$) follicle numbers. γH2AX protein was increased ($P < 0.05$) by both obesity and PM, indicating DNA damage occurrence. mRNA encoding DNA damage repair genes Atm, Prkdc, Parp1, Xrcc6 and Rad51 were not altered by obesity, however, Brca1 was decreased ($P < 0.05$) in ovaries of obese females. PRKDC and XRCC6 protein levels were reduced ($P < 0.05$) in ovaries of obese females.
by obesity while ATM, BRCA1 and RAD51 protein levels were increased ($P < 0.05$) by PM exposure. These results indicate that PM induces ovarian DNA damage as a mode of ovotoxicity, and that the ovary is responsive to such an insult and activates DNA repair proteins in ovaries. Additionally, and of concern, obesity altered the ovarian response to an ovotoxic exposure.

**Introduction**

The mammalian ovary is the female gamete-producing reproductive organ. Primordial germ cells undergo mitosis during oogenesis to establish a gamete pool, arrested in the diplotene stage of meiosis [1]. Females are born with a finite number of gametes, contained in primordial follicle structures, which are required to remain viable over the female reproductive lifetime [1]. Primordial follicles are activated to either develop towards ovulation or undergo atresia, a natural process of elimination [2]. Through this process, females are eventually depleted of all follicles and reproductive senescence ensues [1, 2]. Several factors, including genetics and ovotoxic chemical exposures accelerate loss of follicles [3, 4], which can phenotypically manifest as infertility and premature ovarian failure.

Cyclophosphamide (CPA) is a chemotherapeutic agent used to treat cancer and autoimmune diseases. CPA can induce rapid amenorrhea, potentially due to antral follicle destruction, or premature ovarian failure, attributed to primordial follicle depletion [5]. CPA has also been demonstrated to cause infertility by increasing the number of early growing follicles and decreasing primordial follicle numbers through follicular activation in mouse ovaries [6], a phenomenon previously observed in ovotoxicity induced by 4-
vinylcyclohexene diepoxide [7]. In rodents, species-specific differences in CPA-induced ovarian toxicity are noted, with primordial follicles being most sensitive in mice [8] and antral follicles in rats [9]. Oxidation of CPA by hepatic cytochromes P450 to 4-hydroxycyclophosphamide (4-OH-CPA) involve a cascade of non-enzymatic reactions which ultimately leads to the production of phosphoramide mustard (PM) [10]. PM has been shown to be an active ovotoxic CPA metabolite by destroying both primordial and primary follicles [8, 11]. PM-induced DNA damage represents at least one mechanism by which ovarian follicle loss is caused [12]. PM is known to destroy rapidly dividing cells by covalently binding to DNA, inducing DNA-DNA, DNA-protein cross links and DNA double strand breaks (DSB) [13]. Upon DSB induction, cells activate a DNA damage response (DDR) that comprises cell cycle arrest, DNA damage repair, and subsequent cell cycle resumption [14]. Phosphorylation of the histone H2AX (γH2AX) at Serine 139 is an early event in the DDR, resulting in recruitment and maintenance of DNA repair molecules at the sites of DSB until repair is complete, and is considered a gold standard for DSB localization. γH2AX can be activated by ataxia-telangiectasia mutated (ATM), ATM-related (ATR) and DNA-dependent protein kinases (DNA-PKcs) in response to DSB. Repair of the break can be accomplished through the action of two major pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). The integrity of the gamete genome is critical for the health of offspring. If alterations in DNA of primordial follicles remain, these changes could cause detrimental changes in offspring [12]. This has been evidenced by mice exposed once to CPA which had increased malformations [15]. This is therefore a major concern for women wishing to have children subsequent to cancer treatments [16].
Obesity induces DNA damage in hematopoietic cell transplant recipients that have been treated by CPA [17]. It is known that cellular DNA damage can arise from the action of free radicals and obesity [18]. In addition, high fat diets can accelerate oxidative stress and oxidative DNA damage [19]. BRCA1 and BRCA2 are crucial members of the ATM-mediated DSB repair family of genes. Impairment of BRCA1-related DNA DSB repair was associated with accelerated loss of the ovarian follicular reserve and with accumulation of DSB in human oocytes, suggesting that DNA DSB repair efficiency is an important determinant of oocyte aging in women [20]. Our previous studies have indicated the presence of DNA damage at a basal level in the ovaries of obese mice, and demonstrated increased sensitivity to the ovotoxicant [21], 7,12-dimethylbenz[a]anthracene (DMBA) which, similar to PM, is an alkylating agent. Also, our work using cultured ovarian granulosa cells [22] and an ex vivo ovarian culture system [23], have demonstrated that PM-induced DNA damage as a mode of ovotoxicity. Thus, we hypothesized that PM-induced ovotoxicity would cause DNA DSB’s in vivo, to which the ovary will mount a protective DNA repair response. In addition, we proposed that the ovaries of obese mice would have an altered response to PM exposure.

Methods and Materials

Reagents

PM was acquired from the National Institutes of Health National Cancer Institute (Bethesda, MA). Sesame oil (CAS # 8008-74-0), 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, Ammonium persulphate, Glycerol, N’N’N’N’-Tetramethylethlenediamine (TEMED), Tris base, Tris HCL, Sodium chloride, Tween-20
were purchased from Sigma-Aldrich Inc. (St Louis, MO). RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA) with the exception of the BRCA1(C-20) primary antibody which was from Santa Cruz Biotechnology (Santa Cruz, CA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-mouse and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

**Animals and tissue collection**

Briefly, four week old female wild type normal non-agouti (a/a; designated lean; n = 10) and agouti lethal yellow (KK.Cg-Ay/J; designated obese; n = 10) were purchased from Jackson laboratories (Bar Harbor, ME 002468). All animals were housed in cages under a 12 h light/dark photoperiod with the temperature between 70-73°F and humidity approximately 20-30%. The animals were provided with a standard diet (Teklad 2014 global 14% protein rodent maintenance diet) with *ad libitum* access to food and water until 6, 12, 18 or 24 weeks of age. These animals were part of a previously described project [24]. Tissues were collected when mice were in the pro-estrus stage of their estrous cycle. All animal experimental procedures were approved by the Iowa State University Animal Care and Use Committee.

**In vivo PM exposure and tissue collection**

A separate group of lean and obese mice aged 15 wks of age in both the lean and obese groups were intraperitoneally (i.p) dosed once with sesame oil (SO) or PM (95%; 25 mg/kg)
(n=5/group). This dose was chosen based on the literature [11, 25]. Mice were euthanized 3 days after the end of dosing in their pro-estrus phase. Ovary, uterus, liver and spleen weights were obtained. One ovary from each mouse was fixed in 4% paraformaldehyde and one ovary was preserved in RNA later at -80°C for RNA and protein isolation.

**Ovarian histology and follicle counting**

Ovaries were serially sectioned (5 µM thickness) and every 6th section was mounted and stained with hematoxylin and eosin. Numbers of healthy follicles were classified and counted in every 6th section according to the procedures as previously described [11].

**RNA isolation and qRT-PCR**

Total ovarian RNA was isolated using an RNeasy Mini kit (Qiagen) and the concentration was determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE) (n=3). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). cDNA was diluted (1:20) in RNase-free water. Diluted cDNA (2 µl) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers for **Atm**, **Brcal**, **Prkdc**, **Parp1**, **Rad51**, **Xrcc6** and **Gapdh** were designed by Primer 3 Input Version (0.4.0) [26]. The regular cycling program consisted of a 15-min hold at 95°C and 45 cycles of denaturing at 95°C for 15s, annealing at 58°C for 15s, and extension at 72°C for 20s at which point data were acquired. There was no difference in **Gapdh** mRNA expression between treatments, thus each sample was normalized to **Gapdh** before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method [27, 28].
**Protein isolation and Western blotting**

Total ovarian protein was isolated from randomly chosen animals (n=3) by homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described [31]. Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a standard BCA protocol. SDS-PAGE was used to separate protein homogenates which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 5 % milk in Tris-buffered saline containing tween 20, followed by incubation with one of: anti-rabbit PARP1 antibody (1:200), anti-rabbit phosphorylated H2AX antibody (γH2AX; 1:100), anti-mouse ATM antibody (1:100), anti-mouse RAD51 antibody (1:500), anti-mouse XRCC6 antibody (1:100), anti-rabbit BRCA1 antibody (1:500), or anti-rabbit PRKDC antibody (1:100) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with species-specific secondary antibodies (1:2000-5000) for 1h at room temperature. Membranes were washed 3X in TTBS and incubated in enhanced chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values.

**Statistical analysis**

Raw data for all the experiments were analyzed by unpaired t-test using Graphpad Prism 5.04 software. Different letters indicate $P$-value < 0.05 which was considered a significant difference between treatments. For graphical purposes, protein expression is presented as the mean raw densitometry value ± SE of the respective control.
Results

Effect of PM exposure on body and organ weight

Body weights were obtained prior to PM injection in both lean and obese groups. As expected, body weights were increased ($P < 0.05$) in mice that were grouped as the obese controls (40.8 ± 0.66 g) and obese PM (43.25 ± 2.28 g) subjects compared to those grouped as lean control (34.0 ± 1.81 g) and lean PM (30.40 ± 1.36 g) (Figure 1A).

Ovarian weight was not altered by PM exposure (lean control: 0.0146 ± 0.001 g; lean PM: 0.0125 ± 0.009 g). In contrast, PM exposure decreased ($P < 0.05$) ovarian weight in obese mice (obese control: 0.0131 ± 0.0013 g; obese PM: 0.0106 ± 0.0008 g) relative to their control treated littermates. As previously reported [21] there were no difference in ovarian weight between lean control and obese control ovaries (Figure 1B).

The uterus weight was decreased ($P < 0.05$) in both the obese control 0.0796 ± 0.014 g) and obese PM (0.0610 ± 0.007 g) groups compared to the lean control (0.139 ± 0.024 g) and lean PM (0.110 ± 0.004 g) treated mice. There was no effect of PM exposure on uterine weight (Figure 1C).

Spleen weight was increased ($P < 0.05$) in obese (0.142 ± 0.003 g) compared to lean control subjects (0.123 ± 0.007 g). PM exposure reduced ($P < 0.05$) spleen weight in lean PM (0.095 ± 0.002 g) compared to lean control (0.123 ± 0.007 g) treated mice. PM exposure also reduced ($P < 0.05$) spleen weight in obese PM (0.0795 ± 0.002 g) compared to obese control (0.142 ± 0.003 g) mice (Figure 1D). Liver weight was increased ($P < 0.05$) in obese control (2.362 ± 0.056 g) and obese PM (1.960 ± 0.151 g) compared to lean control (1.290 ± 0.077 g) and lean PM (1.44 ± 0.084 g). PM exposure reduced liver weight in obese PM (1.960 ± 0.151 g) compared to obese control (2.362 ± 0.056 g) (Figure 1F).
Impact of PM exposure on healthy follicle number in lean and obese females

There were reduced ($P < 0.07$) numbers of healthy primordial follicles in ovaries of obese mice (Lean: CT: 91.4 ± 20.56; Obese: 60.0 ± 12.72) relative to lean controls. PM reduced ($P < 0.05$) the number of healthy primordial follicles in both lean (CT: 91.4 ± 20.56; PM: 55.6 ± 15.61) and obese ovaries (CT: 60.0 ± 12.72; PM: 29.5 ± 19.17) compared to lean control (Figure 2A).

PM reduced ($P < 0.05$) the number of healthy primary follicles in both lean (CT: 38.0 ± 4.7; PM: 30.6 ± 3.07) and obese ovaries (CT: 28.2 ± 5.35; PM: 24.25 ± 8.1). There were also reduced ($P < 0.05$) numbers of healthy primary follicles (Lean CT: 38.0 ± 4.7; Obese CT: 28.2 ± 5.35) in obese ovaries relative to lean control (Figure 2B). There was no difference in secondary healthy follicles between the strains or between the treatments (Figure 2C).

Identification of PM-induced ovarian DNA DSB using γH2AX protein

The basal level of γH2AX protein was higher ($P < 0.05$) in ovaries from obese (0.69 ± 0.04) compared to lean control mice (0.59 ± 0.01). However, PM exposure increased ($P < 0.05$) γH2AX protein abundance in both lean (CT: 0.59 ± 0.01; PM: 0.71 ± 0.04) and obese (CT: 0.69 ± 0.04; PM: 0.90 ± 0.05) ovaries (Figure 3A, B).

Investigation of PM exposure effect on Atm mRNA and protein expression

Basal levels of Atm mRNA were increased ($P < 0.05$) in obese control treated mice (0.60-fold ± 0.25) compared to lean control ovaries (1.0-fold ± 0.2). While there was no impact of PM exposure in ovaries from lean mice, an increase in Atm mRNA was observed in ovaries from obese PM-treated mice (1.5-fold ± 0.39) compared to the vehicle control-treated group (0.60-fold ± 0.25) (Figure 4A). Basal levels of ATM protein were higher ($P <$
PM increased ($P < 0.05$) ATM protein abundance in both the lean (0.60 ± 0.01) and obese PM exposed ovaries (0.63 ± 0.01) (Figure 4B, C).

**Effect of PM exposure on Prkdc mRNA and protein expression in lethal yellow mice ovaries**

There were no differences in basal levels of ovarian Prkdc mRNA in obese (0.19-fold ± 0.25) compared to lean (1.0-fold ± 0.10) mice. PM exposure increased ($P < 0.05$) ovarian Prkdc mRNA level in both lean (0.33-fold ± 0.14) and obese (0.32-fold ± 0.10) exposed mice (Figure 5A). PRKDC protein level was lower ($P < 0.05$) in ovaries from obese (CT: 0.52 ± 0.02; PM: 0.56 ± 0.03) compared to lean mice (CT: 0.64 ± 0.02; PM: 0.70 ± 0.03) (Figure 5B, C).

**Impact of PM exposure on Xrcc6 mRNA and protein expression**

Basal levels of Xrcc6 mRNA expression were greatly reduced ($P < 0.05$) in ovaries from obese control (0.70-fold ± 0.05) compared to lean control-treated ovaries (1.0-fold ± 0.13). PM decreased ($P < 0.05$) Xrcc6 mRNA expression in both obese PM (0.78-fold ± 0.02) and lean PM (0.88-fold ± 0.02) ovaries compared to lean control ovaries (Figure 6A). Similarly, ovarian XRCC6 protein level was lower ($P < 0.05$) in obese control (0.49 ± 0.02) relative to lean control-treated ovaries (0.65 ± 0.04). PM exposure increased ($P < 0.05$) ovarian XRCC6 protein abundance in lean mice (CT: 0.65 ± 0.04; PM: 0.90 ± 0.09), but no impact of PM exposure on XRCC6 protein was observed in obese mice (Figure 6B, C).

**Effect of PM exposure on Parp1 mRNA and protein expression in lethal or obese mice**

There were no changes in basal levels of Parp1 mRNA expression in obese control (0.40-fold ± 0.25) compared to lean control (1.0-fold ± 0.10) ovaries. PM decreased ($P < 0.05$) Parp1 mRNA expression in both obese PM (0.82-fold ± 0.02) and lean PM (0.72-fold
± 0.07) treated ovaries compared to the respective control ovaries (Lean: 1.0-fold ± 0.21; Obese 0.40-fold ± 0.18) (Figure 7A). There were no changes in basal abundance of PARP1 protein in obese control (0.69 ± 0.01) compared to lean control (0.69 ± 0.05) ovaries. PM increased ($P < 0.05$) PARP1 protein expression in lean PM exposed ovaries (1.07 ± 0.09) compared to lean control (0.69 ± 0.05) ovaries, but were no changes in PARP1 protein expression due to PM exposure in ovaries from obese mice (Figure 7B, C).

**Evaluation of PM effects on Brca1 mRNA and protein expression and impact of obesity**

There were no differences in the basal level of Brca1 mRNA expression between the lean and obese ovaries. PM increased ($P < 0.05$) ovarian Brca1 mRNA expression in lean mice (1.4-fold ± 0.68) relative to the control ovaries but this increase was absent in ovaries from obese mice (Figure 8A). The basal level of ovarian BRCA1 protein was lower ($P < 0.05$) in obese (0.64 ± 0.01) relative to lean control-treated (0.81 ± 0.03) mice. PM increased ($P < 0.05$) BRCA1 protein level in both lean (1.01 ± 0.05) and obese (0.93 ± 0.08) PM exposed groups compared to their respective control (Lean: 0.81 ± 0.03; Obese: 0.64 ± 0.01) (Figure 8B, C).

**Investigation of PM impact on Rad51 mRNA and protein expression in lean and obese females**

Rad51 mRNA expression was not different in obese ovaries compared to lean ovaries. However, PM exposure decreased ($P < 0.05$) ovarian Rad51 mRNA expression in lean mice (CT: 1.0-fold ± 0.2; PM: 0.49-fold ± 0.10). In contrast, Rad51 mRNA expression was increased ($P < 0.05$) in response to PM exposure in ovaries from obese mice (1.0-fold ± 0.21) compared to lean mice (0.49-fold ± 0.10) (Figure 9A). There was no basal differential RAD51 protein abundance in obese compared to lean ovaries. PM increased ($P < 0.05$)
RAD51 protein level in both lean (0.89 ± 0.06) and obese (0.86 ± 0.03) exposed groups compared to their respective controls (Lean: 0.65 ± 0.01; Obese: 0.65 ± 0.01) (Figure 9B, C).

**Temporal effect of genotypic changes in relation to DNA repair gene abundance**

Since low levels of DNA damage were observed previously [21] and as indicated by increased γH2AX protein in this study, the impact of progressive obesity on induction of the DNA repair response was determined. After 6 wks of age, obese ovaries had increased ($P < 0.05$) expression of a number of DNA DSB repair genes investigated ($Atm$: 0.87-fold ± 0.2; $Brca1$: 0.30-fold ± 0.04; $Xrcc6$: 0.82-fold ± 0.13; $Rad51$: 0.62-fold ± 0.03), compared to the lean mouse strain (Figure 10A). After 12 wks of age, there was no differential expression in any of the DNA DSB repair gene between the mouse strains (Figure 10B). In the mice aged 18 and 24 wks of age, $Brca1$ mRNA abundance was reduced ($P < 0.05$) in ovaries from obese mice (18 wks: 0.37-fold ± 0.11; 24 wks: 0.38-fold ± 0.08) relative to lean mice ovaries (Figure 10C and D).

**Discussion**

This study investigated whether PM-induced DNA DSB is an initiating event to precipitate follicular atresia. We have previously noted that PM activates the DNA DSB repair mechanism in cultured neonatal rat ovaries [23] and in a rat ovarian granulosa cell line [22], which we propose is a logical mechanism to protect cells from PM-induced ovotoxicity. Also, obesity induces ovarian DNA damage in hematopoietic cells after CPA exposure [17], and we have previously demonstrated that progressive obesity induces DNA damage in ovaries [26]. Thus, the primary objective of this study was to investigate the impact of PM
exposure on the DNA damage response in vivo, and secondly to determine any additive impact of obesity on the ovarian protective response to an ovotoxic insult.

We found that ovarian weight was not altered by obesity at the time point chosen, similar to our previously published findings [21]. We discovered that a single dose of PM decreased ovarian weight in obese mice, consistent with a previous study that in utero exposure of CPA reduced ovarian weight in mice [29]. Reduced uterine weights were reported in obese rats during pseudo pregnancy [30], similar to our findings. As expected, obesity increased liver weight which was consistent with another study in which a high fat diet increased liver weights in male wistar rats [31, 32]. Obesity increased spleen weight in this study, as also previously observed in high fat diet fed mice [32]. No major effect of PM on organ weights was observed in the absence of obesity, with the exception of spleen weight which was reduced. A single exposure of CPA (250 mg/kg) was found to increase liver weights after 8 days in CD-1 female mice [12]. In that study, spleen weights were decreased 1 day after exposure, similar to our findings, but were increased 8 days later in mice dosed with 150 or 250 mg/kg CPA [12]. Taken together, PM reduced ovary and spleen weights while obesity altered uterus, spleen and liver weights.

PM is a known ovotoxicant and destroys primordial and primary follicles in mice and rat ovaries both in vitro [22, 33] and in vivo [8, 11, 12]. We found that a single PM dose reduced primordial and primary follicles in lean mice. There were also a lower number of primordial and primary follicles numbers in obese mouse ovaries, and no additive effect of PM was observed. The lower number of follicles due to obesity was in agreement with a previous study from our group which demonstrated that obesity reduced primordial and primary follicle number after 12 weeks of age [24]. Since maintenance of primordial follicles
in a dormant but viable state is essential for normal reproductive activity, obesity-induced reduction of primordial follicle pool could result in premature ovarian failure [1].

Localization of γH2AX protein is considered the gold standard technique for identification of DNA DSB [34]. PM exposure increased γH2AX protein in both lean and obese mice. γH2AX protein recruits DNA repair molecules to the site of damage until repair is complete. Thus, these data corroborate our in vitro data and demonstrate that PM-induced ovotoxicity involves the formation of DNA DSB. We have previously reported [26], and again demonstrate that obesity alone results in a low level of DNA DSB as evidenced by the presence of ovarian γH2AX. We do not know the cause of such DNA DSB in ovaries from obese mice, but speculate that increased reactive oxygen species could be present [35]. Considering that protection of the germline is an evolutionary necessity, we next investigated that ovarian capacity for DNA DSB repair.

ATM is a protein kinase which activates downstream signaling molecules that involved in the DNA repair process. ATM also phosphorylates P53 to initiate cell cycle arrest [36], DNA repair genes [37] and to regulate the expression of a number of apoptosis-related genes [38]. PM exposure resulted in increased Atm mRNA and/or protein abundance in both lean and obese ovaries, again confirming that DNA DSB is formed as a result of ovarian PM exposure. We have previously shown the same effect in two in vitro models; cultured granulosa cells [22] and cultured neonatal rat ovaries [23], thus these data confirm that ATM activation is a downstream event that results from PM exposure. Interestingly, Atm mRNA was increased in the ovaries from obese mice, a response which was absent in ovaries from their lean counterparts. These data indicate that ATM act as an ovarian sensor of DNA DSBs and activates the DNA repair mechanism.
A protein that involved in the NHEJ repair pathway is PRKDC, a DNA dependent protein kinase. PRKDC activates P53 with help of CHK2 upon DNA damage [39]. Prkdc gene expression was increased by PM exposure in both lean and obese ovaries indicating that the NHEJ repair pathway was being activated in PM exposed ovaries. We have previously found the same effect in cultured granulosa cells [22] and neonatal cultured rat ovaries [23] after PM exposure. Interestingly, PRKDC protein was at a lower level in ovaries from obese mice and no increase in PRKDC protein level after PM exposure was observed similar to our previous study from obese mice [26]. Inhibition of PRKDC enhances heat-induced apoptosis independent of heat shock protein in human cervical carcinoma HeLa S3 cells [40], this could contribute to obesity-induced reduced fecundity. These data indicates obesity has a low level of DNA repair by reducing PRKDC protein abundance while the ovary initiates cell survival by inducing the NHEJ repair mechanism in response to PM exposure.

XRCC6 is a KU protein essential for formation of a heterodimer with XRCC5 and is also part of the NHEJ repair pathway. Ovarian XRCC6 protein was increased by PM exposure in lean mice but this response was reduced by obesity. This lower level might be due to basal DNA damage which is present in ovarian tissue during obesity. We have previously found that PM increased XRCC6 protein expression in SIGC cells [22], while a study of progressive obesity showed that obesity decreased XRCC6 protein expression [26]. Decreased XRCC6 representing the defective DNA repair and chromosomal instability has been reported in embryonic cells [41] suggesting that PM activates but obesity attenuates the NHEJ repair mechanism by decreasing XRCC6 protein abundance.

Studies have demonstrated that PARP1 is involved in the regulation of DNA repair and cell survival [42, 43]. PARP1 has a role in repair of single strand DNA breaks (SSBs) and is
also involved in post-translational modification of nuclear proteins during DNA damage and activated cellular process including DNA replication, DNA repair, apoptosis and genome stability [44]. PM exposure decreased Parp1 mRNA expression irrespective of metabolic status, while PARP1 protein was increased only in lean ovaries. Our previous study from SIGC cells found that PM increased PARP1 protein abundance regardless of dose and time of exposure [22] and in cultured neonatal rat ovaries [23]. There were no changes in PARP1 protein level, but PM decreased Parp1 mRNA expression in obese ovaries. In the absence of PARP1, when SSBs are encountered during DNA replication, the replication fork stalls, and DSBs accumulate in synchronized HeLa cells by radiation exposure [45]. These data suggest that absence of PARP1 protein during obesity might contribute to increased levels of DNA damage observed in obese ovaries.

BRCA1 is a protein that repairs DNA DSBs through the HR pathway. Impairment of BRCA1-related DNA DSB repair has been associated with accelerated loss of the ovarian follicular reserve and with accumulation of DSB in human oocytes, suggesting that DNA DSB repair efficiency is an important determinant of oocyte aging in women [20]. PM increased Brca1 mRNA and protein expression while BRCA1 protein was lowered by obesity. This is consistent with our data demonstrating that PM increased BRCA1 protein abundance in cultured neonatal rat ovaries [23] and progressive obesity reduced ovarian BRCA1 protein [26]. The data from this study suggests that BRCA1 is induced as part of the ovarian protective response to PM exposure and that the HR pathway is activated in addition to the NHEJ pathways. In addition, reduction of BRCA1 by obesity may accelerate ovarian follicle loss by reducing the capacity for DNA repair.
RAD51 plays a major role in homologous recombination of DNA DSB repair using sister chromatids [47]. RAD51 protein was increased by PM exposure in both lean and obese ovaries while mRNA abundance was decreased by PM exposure in lean ovaries. RAD51 interacts with BRCA1 during the DDR, and DMBA exposure increased RAD51 protein abundance in both prostate tissue [46] and in SIGC cells in a dose- and time-dependent manner [22] while RAD51 protein level was reduced during the time of follicle loss in neonatal cultured rat ovaries after PM exposure [23]. Also, RAD51 protein level was consistent with BRCA1 expression after PM exposure. These data indicated that RAD51 protein could interact with RAD51 to repair DNA through HR repair mechanism.

Taken together, the data described herein strongly support that PM induces DNA DSB’s in vivo to which the ovary responds by activation of the DDR. In addition, a blunted DDR was observed in ovaries from obese mice, which could contribute to the basal DNA DSB that is apparent in these ovaries. Since a blunted DDR was supported by the data from obese ovarian tissue, we examined basal mRNA abundance of DDR genes during progressive obesity from samples available through another study by our group. *Atm, Brca1, Xrcc6* and *Rad51* mRNA abundance were increased at 6 wks of age, but no other changes with the exception of reduced *Brca1* were noted at other time points examined. This seems paradoxical since γH2AX was observed in the absence of a chemical insult in ovaries from obese mice. It should be noted that the study described only examined a single time point, and perhaps the DDR occurred at a faster rate in ovaries from obese ovaries than those from lean mice, although, the occurrence of γH2AX in obese ovarian tissue does not support this theory.
In conclusion, PM induced ovarian DNA damage with an accompanying increase in the ovarian protective response to counteract such damage. Obesity altered the ovarian response to PM exposure, which could potentially contribute to a poorer reproductive outcome following CPA/PM treatment. Future work directed at preventing PM-induced ovarian DNA damage remains necessary.

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Figure 1. Effect of PM exposure on body and organ weight

Body weights were measured at 15 wks old in both lean and obese group and then mice were intraperitoneally (i.p) dosed with sesame oil (SO) or PM to measure other organ weights. Values are expressed as mean ± SE; n=5. Different letters indicate the statistical difference $P$-value < 0.05. Obesity increases body and liver weight while reduces uterus weight. PM decreases spleen and ovarian weight.
Mice were intraperitoneally (i.p) dosed with sesame oil (SO) or PM to determine healthy follicles. Follicles are classified as (A) primordial follicles; (B) primary follicles; and (C) secondary follicles. Values are expressed as mean ± SE; n=5. Statistical significance was defined as *= P < 0.05 while P < 0.1 was considered a trend towards a difference from control. Both obesity and PM reduces number of healthy follicles.

Figure 2. Impact of PM exposure on healthy follicle number
Figure 3. Identification of PM-induced ovarian DNA DSB using γH2AX protein

After treatment, protein was isolated from ovaries to determine the γH2AX protein expression using Western blotting (A, B). In western blotting, equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Values are expressed as mean ± SE; n=3. Different letters indicate $P < 0.05$. Both obesity and PM increases DNA damage.
Figure 4. Investigation of PM exposure effect on Atm mRNA and protein expression

After treatment, RNA and protein was isolated from ovaries to determine the Atm (A) mRNA and (B, C) protein expression using qPCR and Western blotting, respectively. In qPCR, each sample was normalized to Gapdh before quantification. In western blotting, equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Values are expressed as mean ± SE; n=3. Different letters indicate $P < 0.05$. Both obesity and PM increases DNA damage.
Figure 5. Effect of PM exposure on Prkdc mRNA and protein expression

After treatment, RNA and protein was isolated from ovaries to determine the Prkdc (A) mRNA and (B, C) protein expression using qPCR and Western blotting, respectively. In qPCR, each sample was normalized to Gapdh before quantification. In western blotting, equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Values are expressed as mean ± SE; n=3. Different letters indicate $P < 0.05$. Obesity reduces PRKDC protein level.
After treatment, RNA and protein was isolated from ovaries to determine the *Xrc6* (A) mRNA and (B, C) protein expression using qPCR and Western blotting, respectively. In qPCR, each sample was normalized to Gapdh before quantification. In western blotting, equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Values are expressed as mean ± SE; n=3. Different letters indicate $P < 0.05$. Obesity reduces XRCC6 protein level.
Figure 7. Effect of PM exposure on Parp1 mRNA and protein expression

After treatment, RNA and protein was isolated from ovaries to determine the Parp1 (A) mRNA and (B, C) protein expression using qPCR and Western blotting, respectively. In qPCR, each sample was normalized to Gapdh before quantification. In western blotting, equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Values are expressed as mean ± SE; n=3. Different letters indicate $P < 0.05$. PM increases PARP1 protein level only in lean ovaries.
Figure 8. PM effects on Brca1 mRNA and protein expression and impact of obesity

After treatment, RNA and protein was isolated from ovaries to determine the Brca1 (A) mRNA and (B,C) protein expression using qPCR and Western blotting, respectively. In qPCR, each sample was normalized to Gapdh before quantification. In western blotting, equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Values are expressed as mean ± SE; n=3. Different letters indicate $P < 0.05$. PM increase BRCA1 protein level while obesity reduces in control ovaries.
Figure 9. Investigation of PM impact on *Rad51* mRNA and protein expression

After treatment, RNA and protein was isolated from ovaries to determine the *Rad51* (A) mRNA and (B, C) protein expression using qPCR and Western blotting, respectively. In qPCR, each sample was normalized to Gapdh before quantification. In western blotting, equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values. Values are expressed as mean ± SE; n=3. Different letters indicate $P < 0.05$. PM increases RAD 51 protein levels.
Figure 10. Temporal effect of genotypic changes in relation to DNA repair gene abundance

Ovaries were collected from 6, 12, 18 and 24 wks mice and RNA was isolated to perform qRT-PCR. Values are expressed as mean fold change ± SE; n=3. Statistical significance was defined as * = P < 0.05. DNA repair genes were increases in obese mice at earlier time but no changes at later time point. Brca1 decreases during progressive obesity.
CHAPTER 9. GENERAL DISCUSSION

The female reproductive organ ovary is composed of follicles at different stages of development. Chemicals which selectively damage large growing or antral follicles can cause temporary loss reproductive function while primordial follicle loss can result in permanent infertility and premature ovarian failure. This dissertation research focused on investigating cellular and molecular mechanism of chemical-induced ovarian follicle loss (ovotoxicity). Specifically, mRNA and protein expression profiles of DNA repair, cell cycle arrest, apoptosis and gap junction members were quantified using qRT-PCR, western blotting or immunohistochemistry techniques after exposure to two different chemicals: Phosphoramide mustard (PM) and 7,12-dimethylbenz[a]anthracene (DMBA). Three physiological models were employed 1) spontaneously immortalized rat granulosa cells (SIGC); 2) In vitro ovarian culture system; 3) In vivo obese mice model.

7,12-Dimethylbenz[a]anthracene-Induced Ovotoxicity

DMBA-induced DNA damage and repair mechanism

DMBA is a polyaromatic hydrocarbon liberated from burning of organic matter, including cigarette smoke, charred foods and car exhaust fumes (Gelboin, 1980). DMBA causes destruction of all follicle types leading to premature ovarian failure in mice and rats (Mattison and Nightingale, 1980). For first study, we used an in vitro rat ovarian culture system because this system mimics the effects of ovotoxicant with those reported in vivo (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006). For our second study, we used an in vivo obese mouse model because we wanted to compare the impact of DMBA exposure between in vitro and in vivo systems and also determine if there was elevated levels
of DNA damage due to obesity, based on another study from our group which demonstrated enhanced sensitivity of the ovaries from obese females of DMBA-induced ovotoxicity, likely due to elevated basal levels of microsomal epoxide hydrolase, the enzyme that converts DMBA to the DMBA-3,4-diol-1,2-epoxide metabolite, which has the potential to interact with DNA due to the presence of two epoxide groups thereon (Rajapaksa et al., 2007; Igawa et al., 2009).

Use of a neonatal ovary culture system has determined that a single DMBA exposure depleted large primary follicles at concentrations of 12.5 and 75 nM, while secondary follicles were destroyed at the 12.5 nM concentration only (Madden et al., 2013). We also used the same DMBA concentration for our in vitro ovarian culture system and confirmed that DMBA induced apoptosis at these concentrations by increasing caspase-3 in the ovary. DMBA can cause dose-dependent DNA damage in extra-ovarian tissues (peripheral lymphocytes, liver and skin cells) when exposed along with physical stress in rats (Muqbil et al., 2006). Having confirmed that atresia via apoptosis is ongoing after DMBA exposure; we determined the impact of DMBA on DNA repair proteins as a proxy measurement for DNA damage induction. We localized γH2AX protein to the nucleus and granulosa cells of large primary and secondary follicles after DMBA exposures; however this staining was negligible in the oocyte nucleus of control-treated ovaries. Additionally, γH2AX total protein was increased in ovaries exposed to a concentration of DMBA that causes primordial and small primary depletion after 4 days. Since, detection of γH2AX is considered the gold standard for localization of DNA double strand breaks (DSBs) (Paull et al., 2000; Svetlova et al., 2010) these data indicate that DNA damage is occurring in ovaries exposed to DMBA. DSB in DNA are the most cytotoxic lesions, generated by ionizing radiation, man-made chemicals
(Van Gent et al., 2001) and chemotherapeutic drugs (Helleday et al., 2008). These DSB’s pose a serious threat to genome stability if either unrepaired or repaired incorrectly, and could potentially lead to permanent damage that could be a negative consequence for gamete health (Petrillo et al., 2011). DSB are repaired by two major pathways that are Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ). In this study, mRNA encoding Atm, Xrcc6, Brca1, Rad51 and ATM and PARP1 protein were increased in a dose- and time-dependent manner. This indicates that exposure to DMBA causes ovotoxicity through DSB formation and that the ovary mounts a protective response in order to repair this damage.

For our second study, we employed both lean and obese mice to confirm in vivo DMBA-induced DNA damage and obese mice, since obesity has negative consequences on reproduction such as polycystic ovarian syndrome, menstrual disorders, intrauterine fetal death and infertility (Haslam and James, 2005; Cardozo et al., 2012). Obesity also detrimentally affects pregnancy rates in natural and assisted conception potentially by reducing oocyte quality (Wu et al., 2011). Primordial and small primary follicle numbers were reduced in ovaries from obese mice with a concomitant increase in number of secondary and pre-ovulatory follicles relative to lean mouse ovaries (Nteeba et al., 2014). Also higher basal levels of mEH and greater mEH induction in response to DMBA exposure (Nteeba et al., 2014) indicates that ovarian tissue from obese females could have potentially greater exposure to the ovotoxic metabolite of DMBA (Miyata et al., 1999). We also used the same mouse model for our study to investigate DMBA-induced DNA damage and ovarian DNA repair responses against chemical-induced ovotoxicity. Additionally, since obesity is associated with increased incidence of offspring birth defects, we hypothesized that a
DMBA-induced DNA damage response (DDR) might be compromised in ovarian tissue from obese females. In this study, basal protein abundance of PRKDC and BRCA1 proteins was lower in ovaries from obese females, while higher levels of γH2AX and PARP1 were observed. Impairment of BRCA1-related DNA DSB repair has been associated with accelerated loss of the ovarian follicular reserve and with accumulation of DSB in human oocytes, suggesting that DNA DSB repair efficiency is an important determinant of oocyte aging in women (Titus et al., 2013). Ovarian ATM, XRCC6, PRKDC, RAD51 and PARP1 proteins were increased by DMBA exposure in lean mice. A blunted DMBA-induced increase in XRCC6, PRKDC, RAD51 and BRCA1 was observed in ovaries from obese mice, relative to lean counterparts. This indicates that exposure to DMBA causes ovotoxicity through DSB formation and the ovarian DDR was partially attenuated in obese females raising concern that obesity may be an additive factor during chemical-induced ovotoxicity.

Impact of DMBA exposure on connexin gap junction proteins

The initiating event in DMBA-induced follicle destruction remains unclear; however, since cell to cell communication is vital for follicular viability, it is possible that perturbation to communication within the follicle could have detrimental consequences for the oocyte. CX37, CX43 and CX45 are the major ovarian gap junction proteins, shown to be essential for folliculogenesis and production of fertilizable oocytes (Kidder and Mhawi, 2002). CX37 is located between the granulosa cell and oocyte (Simon et al., 1997), while CX43 and 45 are located between granulosa cells (Granot et al., 2002; Kidder and Mhawi, 2002). Cx37-null mouse oocytes suffer growth retardation and do not survive to become meiotically competent (Carabatsos et al., 2000). Follicle growth is also interrupted: Cx37<sup>−/−</sup> granulosa cells form structures resembling corpora lutea in the absence of ovulation. CX43 levels are increased in
granulosa cells following activation of follicular growth and maturation (Melton et al., 2001), while reduced granulosa cell expression of CX43 is linked to elevated apoptosis in porcine, bovine (Johnson et al., 1999; Cheng et al., 2005) and avian (Krysko et al., 2004) species. Thus, CX37, CX43 and CX45 play important roles in the ovary to maintain follicular and oocyte viability and quality. Little is known about the impact of ovotoxicant exposures on ovarian function, thus in this study we investigated any impact of DMBA on ovarian gap junction genes Cx37, Cx43 and Cx45. We utilized a neonatal rat whole ovary culture and an in vivo obese mouse model to determine the effect of DMBA exposure on gap junction protein expression.

In the neonatal rat ovarian culture system, a single DMBA exposure at two concentrations: 12.5 nM and 75 nM were used to determine the effect of DMBA on Cx37 and Cx43 mRNA and protein level. These exposures have previously been shown to cause large primary and secondary follicle loss (Madden et al., 2013). In addition, we have previously shown that these concentrations induce DNA damage and increased caspase 3 levels after 8 days of exposure. Our hypothesis was that altered Cx37 and Cx43 at time points prior to observed follicle loss may support that they are targets for DMBA as part of DMBA’s mechanism of ovotoxicity. In this study, CX37 and CX43 protein were increased at 4 days of DMBA exposure while DMBA decreased CX37 and CX43 protein after 8 days. DMBA is proposed to induce apoptosis in granulosa and theca cells of pre-ovulatory follicles through generation of reactive oxygen species (ROS), leading to increased expression of pro-apoptotic BAX, activation of the executioner CASP3 and ultimately cell death (Tsai-Turton et al., 2007). In support of this, BAX-deficient mice ovaries are resistant to DMBA-induced primordial follicle destruction (Matikainen et al., 2001). We found mRNA expression of pro-
apoptotic p53 was decreased but no changes in Bax expression were observed after 4 days while increased p53 and Bax mRNA levels were found after 8 days of DMBA exposures. These findings support that DMBA exposure impacts ovarian Cx37 and Cx43 mRNA and protein prior to follicle loss.

**Impact of obesity and DMBA on gap junction protein expression**

Approximately one-third of adults in the USA are obese (Flegal K, Meeker et al., 2010; Meeker et al., 2010), and negative female phenotypic associations include polycystic ovarian syndrome, menstrual disorders, intrauterine fetal death and infertility (Haslam and James, 2005; Cardozo et al., 2012). Obesity also detrimentally affects pregnancy rates in natural and assisted conception potentially by reducing oocyte quality (Wu et al., 2011). Primordial and small primary follicle numbers were reduced in ovaries from obese mice with a concomitant increase in number of secondary and pre-ovulatory follicles relative to lean mouse ovaries (Nteeba et al., 2014). Additionally, ovaries from mice fed a high fat diet showed increased accumulation of endoplasmic reticulum stress, decreased mitochondrial activity and increased apoptosis of cumulus oocyte complexes and ovarian cells (Wu et al., 2010). Interestingly, a high fat diet also reduced cardiovascular connexin expression in female rats resulting in increased risk of ventricular arrhythmia (Aubin et al., 2010). This suggest that obesity have impact on Cx gene mRNA and protein levels, thus this study investigated the impact of obesity on ovarian Cx mRNA and protein levels using the lethal yellow mouse model of progressive obesity. For this study ovaries were collected from lean and obese mice aged 6, 12, 18, or 24 wks. Cx43, Cx45 mRNA and protein levels were decreased after 18 wks while Cx37 mRNA and protein levels were decreased after 24 wks in obese ovaries. These data support that obesity temporally alters gap junction protein in ovaries in a manner that
could contribute to compromised reproduction observed during these physiological paradigms.

Cx37 mRNA and antral follicle protein staining intensity were reduced by obesity while total CX43 protein was reduced in DMBA exposed obese ovaries. Cx43 mRNA and total protein levels were decreased by DMBA in both lean and obese ovaries while basal protein staining intensity was reduced in obese controls. Cx45 mRNA, total protein and protein staining intensity level were decreased by obesity. These data support that obesity temporally alters gap junction protein expression and that DMBA-induced ovotoxicity may involve reduced gap junction protein function. It is possible that such interference in communication between follicular cell types is detrimental to follicle viability, and may play a role in DMBA-induced follicular atresia.

**Phosphoramide Mustard-Induced Ovotoxicity**

**PM-induced DNA adduct formation and DNA damage repair responses**

Phosphoramide mustard (PM), the ovotoxic metabolite of the anti-cancer agent cyclophosphamide (CPA), destroys rapidly dividing cells by forming NOR-G-OH, NOR-G and G-NOR-G adducts with DNA, potentially leading to DNA damage (Cushnir et al., 1990; Johnson et al., 2011). PM causes primordial and small primary follicle loss and induces ovarian DNA damage in rat ovaries (Petrillo et al., 2011). The granulosa cell is the somatic cell component of the oocyte-containing follicle, and close association between the granulosa cell and oocyte is required for follicular development. Loss of granulosa cells during pre-antral and antral stages of follicular development leads to a premature reduction in female fecundity through reduced follicle health and oocyte viability (Walters et al., 2012). An *in
vivo study demonstrated that the destruction of granulosa cells by CPA potentially occurs through apoptosis in rats (Lopez and Luderer, 2004). Since PM-induced DNA damage could predispose the follicle towards atresia, this study investigated whether PM exposure to granulosa cells results in PM-DNA adducts formation and subsequent DDR induction. To investigate whether PM induces DNA adduct formation, DNA damage and induction of the DDR, rat spontaneously immortalized granulosa cells were treated with PM at different time points and concentrations to isolate DNA, RNA and protein to perform mass spectrometry, qRT-PCR, immunofluorescence and western blotting techniques. In this study, cell viability was reduced after 48 h of exposure PM while DNA adducts was detected earlier than cell death. γH2AX, a marker of DNA double stranded break occurrence, was also increased by PM exposure, coincident with DNA adduct formation. Additionally, induction of genes and proteins involved in DNA repair were observed in both a time- and dose-dependent manner. These data support that PM induces DNA adduct formation in ovarian granulosa cells, induces DNA damage and elicits the ovarian DNA repair response.

**Mechanism of PM-induced ovarian follicle loss**

PM causes dose- and time-dependent primordial and primary ovarian follicle loss following DNA damage in mice and rat oocytes (Petrillo et al., 2011). DSBs and DNA replication blocking lesions are apoptotic DNA lesions and, therefore, cells are equipped in detecting these lesions upon formation. Recognition of these DNA lesions starts a protein cascade, which finally results in cell cycle arrest and DNA repair (Zhou and Elledge, 2000). If DNA repair fails, or is overwhelmed by too many DNA lesions, these sensors initiate apoptosis (Friedberg, 2003). To investigate the mechanism of PM induced ovarian follicle loss we used a neonatal rat ovarian culture system and ovaries were exposed to 60 μM of PM.
for 4 days. PM caused primordial, small primary, large primary and secondary follicles after 4 days of exposure (Madden et al., 2014). PM activated DNA repair genes after 12 h of exposure which indicated that PM induces DNA damage earlier than follicle loss. PM activates the DDR genes after 24 h and proteins ATM, PARP1, E2F1, P73 and CASP3 after 96 h of exposure; however PM decreases RAD51 and BCL2 proteins after 96 h of exposure. DNA repair genes are activated earlier than apoptotic genes and proteins and number of DNA repair genes expressed during follicle loss was reduced. Although other studies have found that CPA or PM induces apoptosis to cause cell death (Chen et al., 1994; Schwartz and Waxman, 2001), this study found that PM activates Bbc3 and Bax and decreases BCL2 to cause apoptotic cell death. Also, PM-induced apoptosis is not occurring via P53 dependent pathway but through the E2F1-P73 dependent mitochondrial apoptotic pathway.

The main players of DNA damage recognition are ATM, ATR and DNA-PK, which phosphorylate a multitude of proteins and thus induce the in which P53 and BRCA1/2 play important roles (Roos and Kaina, 2006). ATM is implicated in three crucial functions: regulation and stimulation of DSB repair, activation of cell cycle checkpoints, and signaling to apoptosis. Therefore, they are key nodes in making the decision between survival and death following genotoxin exposure (Roos and Kaina, 2012). To determine the role of ATM during PM exposure we used the ATM inhibitor KU-55933. ATM inhibition prevented loss of primordial, large primary and secondary follicles. Also, small primary numbers were not completely restored to those of control but PM-induced small primary follicle depletion was partially attenuated. This result was similar to a previous study showing that ATM inhibitor improved oocyte survival after doxorubicin exposure (Soleimani et al., 2011) by activation of c-AbI-Tap63 pathway. This indicates that ATM has a pro-apoptotic function in the DNA
damaged ovaries, which was coincident with ATM's role in neurons of developing mouse central nervous system (Herzog et al., 1998). Blocking of ATM activity can cause induction of ATM protein, which might be regulated by P73 and E2fl transcriptional level (Khalil et al., 2012). These results are in correlation with our findings in the ovary, and suggest that ATM serves to eliminate cells with excessive DNA damage by acting on developmental survival checkpoint (Herzog et al., 1998).

Protein kinase c (PKC) is another serine-threonine kinases expressed in rat ovary (Peters et al., 2000), activated by diverse stimuli and participates in cellular process such as growth, differentiation and apoptosis (Hug and Sarre, 1993). A recent study showed that the catalytic fragment of PKCδ interacts with P73β and phosphorylates the transactivation and DNA binding domains of P73β to induce apoptotic function (Ren et al., 2002). However, PKCδ also enhances proliferation and survival of murine mammary cells (Grossoni et al., 2007). This indicates that PKCδ is involved in both apoptotic and anti-apoptotic activities. To investigate the role of PKCδ in neonatal ovaries during PM exposure, we used a PKCδ knockout mice model and cultured ovaries from postnatal day 4 females.

PKCδ knockout mice had reduced number of all stage ovarian follicles compared to wild type ovaries. When these mice were exposed to PM, the number of healthy follicles was reduced by PM but the percentage decline did not differ from the WT ovaries, indicating lack of an additional impact of PKCδ deficiency with PM-induced ovotoxicity. These data do support that PKCδ is essential for ovarian follicle survival which is correlated with previous study that PKCδ enhances proliferation and survival of murine mammary cells (Grossoni et al., 2007) and human breast cancer cell lines (McCracken et al., 2003).
In conclusion, PM induces DNA damage as an upstream event of PM-induced ovotoxicity. Ovary activates DNA repair mechanism at earlier time points to protect against PM-induced follicle loss. However, the repair mechanisms were reduced and cell death mechanisms were overwhelmed at the time of follicle loss. PM-induced apoptosis is not happening by P53 dependent pathway but it occurs through E2F1-P73 dependent mitochondrial apoptotic pathway. Results from PKCδ mice indicated that PKCδ is essential for survival of ovarian follicles and ATM inhibitor improves the survival of ovarian follicles during PM exposure. Although ATM inhibition sensitizes cancer cells to genotoxic agents, the mechanism of ATM regulation might be useful for making more accurate tactics for modulation of ATM activity in cancer therapy.

**Impact of obesity on PM-induced ovotoxicity**

Obesity induces DNA damage in hematopoietic cell transplant recipients that have been treated by CPA (Johnson *et al.*, 2012). It is known that cellular DNA damage can arise from the action of free radicals and obesity (Gursatej Gandhi, 2012). In addition, high fat diets can accelerate oxidative stress and oxidative DNA damage (Djuric and Kritschevsky, 1993). BRCA1 and BRCA2 are crucial members of the ATM-mediated DSB repair family of genes. Impairment of BRCA1-related DNA DSB repair was associated with accelerated loss of the ovarian follicular reserve and with accumulation of DSB in human oocytes, suggesting that DNA DSB repair efficiency is an important determinant of oocyte aging in women (Titus *et al.*, 2013). Our previous studies have indicated that presence of DNA damage at a basal level in the ovaries of obese mice, and demonstrated increased sensitivity to another ovotoxicant DMBA (Nteeba *et al.*, 2014) which, similar to PM, is also an alkylating agent. Also, our work using cultured ovarian granulosa cells (Ganesan and
Keating, Under Review) and an *ex vivo* ovarian culture systems (Ganesan and Keating, Under Review) have demonstrated that PM-induced DNA damage as a mode of ovotoxicity. Thus, we hypothesized that PM-induced ovotoxicity would cause DNA DSB’s *in vivo*, to which the ovary will mount a protective DDR. In addition, we proposed that the ovaries of obese mice would have an altered response to PM exposure. As expected, obesity increased liver weight which was consistent with another study in which a high fat diet increased liver weights in male wistar rats (DeLany *et al.*, 1999; Milagro *et al.*, 2006). Obesity increased spleen weight in this study, as was previously observed in high fat diet fed mice (DeLany *et al.*, 1999). PM exposure reduced spleen weight regardless of body composition, however, decreased ovarian weight was only observed in the obese females exposed to PM. Exposure to PM decreased primordial and primary follicle number which was similar to a previous study from mice and rats (Desmeules and Devine, 2006; Petrillo *et al.*, 2011). γH2AX protein was increased by both obesity and PM, indicating DNA damage occurrence. mRNA encoding DNA damage repair genes *Atm, Prkdc, Parp1, Xrcc6* and *Rad51* were not altered by obesity, however, *Brca1* mRNA abundance was decreased in ovaries of obese females. This seems paradoxical since γH2AX was observed in the absence of a chemical insult in ovaries from obese mice. PRKDC and XRCC6 protein levels were reduced by obesity while ATM, BRCA1 and RAD51 protein levels were increased by PM exposure. These results indicate that PM induces ovarian DNA damage as a mode of ovotoxicity, and that the ovary is responsive to such an insult and activates DNA repair proteins in ovaries. Additionally, and of concern, obesity altered the ovarian response to an ovotoxic exposure which could potentially contribute to a poorer reproductive outcome following CPA/PM treatment. Future work directed at preventing PM-induced ovarian DNA damage remains necessary.
Future Directions

Though, these studies have found novel results, it is necessary to note that they also generated important questions for future research. From our data we demonstrated that chemical-induced DNA damage is an upstream event for follicle loss and the ovary activates DNA repair mechanisms to prevent follicle loss using in vitro and in vivo models. We did not look at the impact of chemical exposure in the offspring of exposed females. So, future studies will need to address the impact of these chemicals after in utero maternal exposure.

Although, the impact of DMBA exposure on gap junction proteins was investigated, we did not examine the effect of PM on ovarian gap junction proteins. Since we found a “gap” between the oocyte and granulosa cells in hematoxylin and eosin stained ovaries after PM exposure, and use of electron microscopy to examine PM-exposed ovaries indicated loss of granulosa cells prior to death of the oocyte (Madden and Keating, under review), these lend support to determining the impact of PM exposure on gap junction protein communication.

Even though, ovotoxicant chemical-induced DNA damage was partially repaired by activation of ovarian repair mechanisms, the unrepaired or mis-repaired DSBs can culminate into severe mutagenic changes in the surviving primordial follicles. Though clinical data do not suggest an increase in the incidence of genetic disorders in children born to cancer survivors (Green et al., 2002), studies from rodent models showed that single dose of doxorubicin treatment increased rates of neonatal death, physical malformations, and chromosomal abnormalities in up to 6 generations of offspring. So, it is very important to study the effect of accumulation of DSBs in ovarian follicles using both in vitro as well as in vivo model.
In our study, ATM inhibition rescued only small follicles but not larger follicles during PM exposure, suggest that some of the follicles are sensitive to PM-induced activation of ATM and some are not sensitive. If such individual differences exist, exploration of factors regulating DSB repair in oocytes will develop a new strategies to protect oocytes by enhancing DNA repair efficiency and preserve fertility in the face of chemotherapy. Also, we are not sure, whether exposure of PM along with ATM inhibited oocytes are good quality or not and studying the quality of oocytes after this exposure will be beneficial too.

**Overall Summary**

The data presented herein demonstrates that ovotoxicants induce DNA damage in both oocytes and in granulosa cells. If this DNA damage passes to next generation it may cause abnormalities in offspring. The ovary activates DNA repair responses to protect against chemical-induced ovotoxicity. These responses are active at lower doses and at earlier time points and the cell death response is activated at later times and at higher doses. Also, gap junction proteins are altered by ovotoxicant chemical exposures studied as part of this work. Obesity had impact on follicular development by interfering with gap junction protein levels and also reduced the DNA repair mechanism after chemical exposure. These findings suggest that ovaries from obese females are more vulnerable to chemical-induced toxicity. Finally understanding the mechanisms of chemical-induced follicle loss will provide therapeutic importance to prevent chemical-induced infertility. This research is not only of benefit for the female themselves, but is also aimed at minimizing damage to female eggs, which could potentially result in negative consequences for the offspring. Ultimately, the goal of this research is to improve reproductive health of both human and animals.
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prepubertal porcine ovaries. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 130, 43-55.


Figure 18. Concentration dependent PM-induced follicle loss

PND4 Fisher 344 rat ovaries were cultured for 8 d in medium containing vehicle control (CEZ) ± PM (30, 45 and 60 µM). Following culture, follicles were counted between the treatments and analyzed. Total number of primordial (A), Small primary (B), large primary (C) and Secondary follicles (D) were expressed as mean raw data ± SE; n=5. Statistical significance was defined as * = P value 0.01, ** = P value 0.001, *** P value 0.0001.
Figure 2. Localization of γH2AX protein after PM exposure

PND4 Fisher 344 rat ovaries were cultured for 8 d or 1 d in medium containing vehicle control (CEZ) ± PM (30 or 60 µM). Immunofluorescence staining was used to determine localization and staining intensity for γH2AX proteins (A). Images were taken using a Leica fluorescent Microscope and number of oocytes with foci (B) and over al foci for γH2AX were analyzed using ImageJ software (NCBI). (n=3 ovaries; 4 section/ovary).
Figure 3. Validation of RT² Profiler PCR array results using qRT-PCR

Fisher 344 rat ovaries were cultured for 2 or 4 d in media containing vehicle control ± 30 µM PM. RNA was isolated and used to perform qRT-PCR. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05.
Figure 4. PM alters DNA repair protein expressions against CEZ

Fisher 344 rat ovaries were cultured for 2 or 4 d in media containing vehicle control ± 30 µM PM and protein was isolated to perform western blot. Results were normalized to Ponceau S and expressed as mean raw data ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = P < 0.05.
Table 1. PM alters DNA damage response genes against CEZ

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<td>1</td>
<td>0</td>
<td>1</td>
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Fisher 344 rat ovaries were cultured for 2 or 4 d in media containing vehicle control ± 30 μM PM. RNA was isolated and used to perform a RT² Profiler PCR array. Values represent fold-change ± SEM relative to a control value of 1 (10 ovaries per pool), normalized to Actb. * = different from control, $P < 0.05$. 
Table 2. Effect of DMBA on glucose metabolism gene expressions

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>p-value</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acly</td>
<td>ATP citrate lyase</td>
<td>0.059539</td>
<td>0.4796</td>
</tr>
<tr>
<td>Aco1</td>
<td>Aconitase 1, soluble</td>
<td>0.172505</td>
<td>0.0195</td>
</tr>
<tr>
<td>Aco2</td>
<td>Aconitase 2, mitochondrial</td>
<td>0.0831</td>
<td>0.6598</td>
</tr>
<tr>
<td>Agl</td>
<td>Amylo-1,6-glucosidase, 4-alpha-glucanotransferase</td>
<td>0.119533</td>
<td>0.3384</td>
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<tr>
<td>Aldoa</td>
<td>Aldolase A, fructose-bisphosphate</td>
<td>0.137154</td>
<td>1.4273</td>
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<tr>
<td>Aldob</td>
<td>Aldolase B, fructose-bisphosphate</td>
<td>0.009347*</td>
<td>0.0166</td>
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<tr>
<td>Aldoc</td>
<td>Aldolase C, fructose-bisphosphate</td>
<td>0.13543</td>
<td>1.7053</td>
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<tr>
<td>Bpgm</td>
<td>2,3-bisphosphoglycerate mutase</td>
<td>0.022937*</td>
<td>0.0902</td>
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<tr>
<td>Cs</td>
<td>Citrate synthase</td>
<td>0.06818</td>
<td>0.5797</td>
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<tr>
<td>Dlat</td>
<td>Dihydrolipoamide S-acetyltransferase</td>
<td>0.06307</td>
<td>0.4244</td>
</tr>
<tr>
<td>Dld</td>
<td>Dihydrolipoamide dehydrogenase</td>
<td>0.003478*</td>
<td>0.4644</td>
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<tr>
<td>Dlst</td>
<td>Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)</td>
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<tr>
<td>Eno1</td>
<td>Enolase 1, (alpha)</td>
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<td>Eno2</td>
<td>Enolase 2, gamma, neuronal</td>
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<tr>
<td>Eno3</td>
<td>Enolase 3, beta, muscle</td>
<td>0.114727</td>
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<tr>
<td>Fbp1</td>
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<td>Fructose-1,6-bisphosphatase 2</td>
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<td>0.4024</td>
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<tr>
<td>Galm</td>
<td>Galactose mutarotase (aldose 1-epimerase)</td>
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<td>Gapdh</td>
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<td>1.234</td>
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<td>Gapdhs</td>
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<td>Gck</td>
<td>Glucokinase</td>
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<td>Gpi</td>
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<td>Benjamini-Hochberg Correction</td>
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<td>Glycogen synthase 2</td>
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<td>Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)</td>
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<td>Hk2</td>
<td>Hexokinase 2</td>
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<td>Hk3</td>
<td>Hexokinase 3 (white cell)</td>
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<td>Hprt1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
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</table>
Following 4 d of culture, RNA was isolated from PND4 rat ovaries exposed to control (CT), 12.5 nM DMBA. RNA was isolated and used to perform a RT² Profiler PCR array. Values represent fold-change ± SEM relative to a control value of 1 (10 ovaries per pool), normalized to Actb. * = different from control, $P < 0.05$. 

<table>
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<td>Ldha</td>
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<tr>
<td>Rplp1</td>
<td>Ribosomal protein, large, P1</td>
<td>0.029232*</td>
<td>0.3291</td>
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