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Accelerated Ovarian Failure: a novel, chemically-induced animal model of menopause

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Abstract

Current rodent models of menopause fail to adequately recapitulate the menopause transition. The intact aging model fails to achieve very low estrogen levels, and the ovariectomy model lacks a perimenopause phase. A new rodent model of Accelerated Ovarian Failure (AOF) successfully replicates human perimenopause and postmenopause, including estrous acyclicity and fluctuating, followed by undetectable, estrogen levels, and allows for the dissociation of the effects of hormone levels from the effects of aging. In this model, an ovotoxic chemical, 4-vinylcyclohexene diepoxide (VCD), selective for primary and primordial follicles, is injected intraperitoneally in animals for 15 days. As the mature follicle population is depleted through natural cycling, ovarian failure follows increasing periods of acyclicity. Administered at low doses, VCD specifically causes apoptotic cell death of primordial follicles but does not affect other peripheral tissues, including the liver and spleen, nor does it cross the blood-brain barrier. In addition to reducing confounds associated with genetic and surgical manipulations, the AOF model maintains the presence of ovarian tissue which importantly parallels to the menopause transition in humans. The VCD injection procedure can be applied to studies using transgenic or knock-out mice strains, or in other disease-state models (e.g., ischemia, atherosclerosis, or diabetes). This AOF model of menopause will generate new insights into women's health particularly in determining the critical periods (i.e., a window of opportunity) during perimenopause for restoring ovarian hormones for the most efficacious effect on memory and mood disorders as well as other menopausal symptoms.

Keywords

estrogen; hormone replacement therapy; perimenopause

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1. INTRODUCTION

Ovarian hormones shape women's health throughout the lifespan. During the last half of women's lives, a decline in ovarian hormones contributes to physiological and psychological aging associated with menopause (for review see Morrison et al., 2006). The menopause transition, or perimenopause, is defined by several stages that precede amenorrhea and postmenopause. Perimenopause usually occurs between ages 45 and 54 and is a time of disrupted hormone levels, including prolonged estrogen withdrawal and decreased fertility (for review see Harsh et al., 2009; Nejat and Chervenak, 2010). In addition to physiological symptoms (i.e., hot flashes and night sweats), a major complaint of menopausal women is neurological changes, especially those related to depression and memory.

Menopause, particularly the perimenopause phase, has been associated with decline in memory functions. Women self-report decreased concentration and poor memory during perimenopause, and decreases in episodic memory also have been reported during perimenopause (Greendale et al., 2009; Joffe et al., 2006). Several clinic-based studies have found that short-term estrogen replacement can improve measures of verbal learning and memory in perimenopausal women, suggesting that declining ovarian estrogens are involved (Sherwin, 2003). Observational studies also suggest that declining ovarian function increases the risk of dementia and mild cognitive impairments (Morrison et al., 2006). For a review of cognitive effects of menopause, hormone replacement, and oophorectomy, see Rocca et al. (2010) in this issue.

Over the past several decades, studies of menopause and hormone replacement therapy have led to a great deal of confusion about their effects on women's health. In particular, the evidence for a neuroprotective role of hormone replacement therapy for cognitive disorders in menopausal women has been mixed, largely due to both experimental confounds and studies limited to postmenopausal women (Morrison et al., 2006; Rocca et al., 2010). Nevertheless, recent studies have shown that hormone replacement therapy initiated during perimenopause lowers the risk of dementia and has cognitive benefits, especially to verbal memory and other hippocampal mediated memory processes (Henderson et al., 2005; Maki, 2006; Zandi et al., 2002). Thus, recent studies have focused on the perimenopause as a "Window of Opportunity" for women to benefit from hormone replacement (Morrison et al., 2006; Rocca et al., 2010).

Although the current rodent models of menopause have and will continue to inform our understanding of the role of both estrogen and hormone replacement therapy in the menopause transition, they fail to adequately recapitulate perimenopause. The intact aging model fails to achieve very low estrogen levels, and the ovariectomy model lacks a transition to perimenopause. A new rodent model of Accelerated Ovarian Failure (AOF) successfully replicates human perimenopause and postmenopause, including estrous acyclicity and fluctuating, followed by undetectable, estrogen levels, and allows for the dissociation of the effects of hormone levels from the effects of aging (Mayer et al., 2004; Mayer et al., 2005). As reviewed here, this novel rodent model of menopause more closely parallels the menopause transition and postmenopause years. The AOF menopause model will generate new insights into women's health particularly in determining the critical periods during perimenopause for restoring ovarian hormones for the most efficacious effect on memory and mood disorders.

2. MODELS OF MENOPAUSE

Although the current rodent models of menopause have furthered our understanding of the role of estrogen in peri- and postmenopause, two most commonly used models (i.e., intact aging and ovariectomy) are lacking in two important aspects of human menopause: very low

estrogen levels and a menopause transition. Although the intact aging model is similar to human menopause with hormonal fluctuations during the menopause transition, it lacks a true menopause (very low to undetectable estrogen levels), and rodents instead go into an estropause with low, but persistent estrogen levels (Maffucci and Gore, 2006). The ovariectomy (OVX) model reduces estrogen to undetectable levels, but by definition lacks the menopause transition.

2.1 Intact Aging Model

Like women, rodents experience natural hormonal fluctuations that occurring in middle age, however, rodents enter an estropause rather than a true menopause (Maffucci and Gore, 2006). During human menopause, estrogen levels are very low or undetectable, progesterone levels decrease, and follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels are elevated (as reviewed in Nejat and Chervenak, 2010; Schmidt and Rubinow, 2009;). Rodents begin to exhibit irregular acyclicity at middle age (9-12 mo), usually characterized by additional days of diestrus I within the normal cycle, resulting in prolonged cycles (Spencer et al., 2008). During this presumptive perimenopause, there is an attenuation and delay of the LH surge and a decrease in fertility, similar to humans (Rubin, 2000; Williams, 2005). Around one year of age rodents enter a state of persistent estrus, during which reproductive cycles cease altogether, or presumptive postmenopause (Frick, 2008). Eventually, as aging progresses to about 16-18 months, rodents transition into an acyclic, anestrus state, in which persistent estrus is observed, ovulation ceases and estrogen as well as other ovarian hormone levels decline from pre-estropausal levels (Rousseau, 2006). Estropausal estrogen and progesterone levels in rats are not as reduced as in mice or humans (as reviewed in Chakraborty and Gore, 2004; Maffucci and Gore, 2006). Whereas rats retain a larger number of primary follicles, mice have complete follicular exhaustion at 24 months of age and demonstrate a greater decrease in estrogen levels than rats in estropause (Chakraborty and Gore, 2004; Maffucci and Gore, 2006). Both mice and rats demonstrate delayed and attenuated gonadotropin surges (Chakraborty and Gore, 2004; Maffucci and Gore, 2006; Rubin, 2000).

Overlapping with these menopausal changes are aged-related changes in other hormones and growth factors. While the intact aging model allows for the retention of ovarian tissue and a substantial transitional period, the hormonal milieu does not reflect what occurs in human menopause, and age related changes present an additional confound (Chakraborty and Gore, 2004; Maffucci and Gore, 2006; Rubin, 2000). Humans spend nearly 50% of their lifespan in reproductive senescence, much more than most other species, and this further confounds the role of hormones and aging the postmenopausal changes (Maffucci and Gore, 2006).

2.2 Ovariectomy Model

The OVX model of menopause is well established in the field of aging (Maffucci and Gore, 2006). In this model, rodents may be OVXed at ages correlating with different life stages. For example, rodents may be OVXed at 2-6 months with regular estrous cycles, 11 months at the beginning of acyclicity, or 18 months at the beginning of constant estrus to model specific aspects of aging (Chakraborty and Gore, 2004; Lekontseva et al., 2010; Maffucci and Gore, 2006; Smith et al., 2010;). Often studies involve steroid replacement following OVX (for review Chakraborty and Gore, 2004; Maffucci and Gore, 2006; Rocca et al., 2010). In one well-accepted paradigm, two weeks after OVX, the rodents receive constant estrogen replacement using silastic capsules containing 5% 17 β -estradiol in cholesterol for a specified period of time (e.g., 3 days) (Gore et al., 2002). However, there are many variations on this paradigm depending on the question being investigated (Chakraborty and Gore, 2004; Maffucci and Gore, 2006). Additionally, steroids can be administered via

silastic capsules, subcutaneous injection, or commercially-available, slow-release pellets (Cohen and Milligan, 1993).

While the OVX model parallels surgically-induced menopause in humans and provides insight in the role of estrogen in the measured responses, it does have two major drawbacks. First, since nearly 90% of menopausal women retain their ovaries, OVXed rodents do not model natural, transitional menopause (Acosta et al., 2010; Shuster et al., 2010). In the OVX model, ovarian hormones are removed abruptly while in natural menopause there is a gradual alteration of hormones that start to occur at perimenopause and change over this transition prior to reaching postmenopause (for review see Nejat and Chervenak, 2010). Second, in addition to estrogens, OVX depletes many other hormones that likely have important roles in menopause and activation affects on the brain (Chakraborty and Gore, 2004; Maffucci and Gore, 2006; Rocca et al., 2010). In particular, the central regulators of estrogens, LH, gonadotropin-releasing hormone (GnRH), and FSH are depleted (Chakraborty and Gore, 2004; Maffucci and Gore, 2006; Rocca et al., 2010; Wise et al., 2002). The removal of ovaries also fails to model levels of androgens produced by residual ovarian tissue (Davison et al., 2005; Fogle et al., 2007; Havelock et al., 2006; Laughlin et al., 2000; Mayer et al., 2004; Rivera et al., 2009).

In the OVX model of menopause, three issues complicate interpretation of data: 1) age at OVX; 2) duration of steroid replacement; and 3) changes in steroid responsiveness (Chakraborty and Gore, 2004; Maffucci and Gore, 2006). Young and old animals respond differently to OVX. Younger animals demonstrate a more rapid and robust decrease in circulating ovarian hormone levels compared to older animals (Chakraborty and Gore, 2004; Maffucci and Gore, 2006). For a given effect (e.g., physiologic level) of exogenous hormone replacement (e.g., estradiol), younger animals require shorter treatment periods and lower doses (for review see Chakraborty and Gore, 2004; Maffucci and Gore, 2006). Variations in the age at OVX, time elapsed since OVX, and in estrogen dose, delivery, and duration, make it difficult to compare across experiments (Chakraborty and Gore, 2004; Maffucci and Gore, 2006; Rocca et al., 2010). Few studies have addressed these issues, but it is clear that estrogen sensitivity decreases when greater time has lapsed since OVX or with increasing age (Morrison et al., 2006; Smith et al., 2010). Studies using the OVX model highlight the estrogen sensitivity of cognitive impairment reported by women who undergo surgical menopause before their natural menopause; they do not shed light on the progressive changes in estrogen responsiveness that occur when the natural cycle of circulating estrogens are disrupted by the onset of menopause.

2.3 Accelerated Ovarian Failure Model of transitional menopause

Recently, Mayer and Hoyer have developed an alternate rodent model of menopause that has been applied to cardiovascular research (Mayer et al., 2004; Mayer et al., 2005; Williams, 2005). For this model, 4-vinylcyclohexene diepoxide (VCD) is injected into mice to selectively accelerate the natural loss of small primordial and primary follicles (for mechanistic reviews see Hoyer and Sipes, 1996; Hoyer et al., 2001; Hoyer and Sipes, 2007). Keating et al. (Keating et al., 2009) demonstrated the involvement of phosphatidylinositol-3 kinase (PI3) signaling in the initiation of VCD-induced ovotoxicity. Keating's work suggests that small, primary follicles are sensitive to VCD exposure and that the decrease in the primordial follicle population is due to increased primordial recruitment to the pool of small, primary follicles via a PI3 signaling pathway (Keating et al., 2009). A recent publication suggests that the direct molecular target of VCD may be a post-translational PI3 signaling pathway in the oocyte itself, as evidenced by changes to the signaling cascade occurring prior to follicular loss (Keating et al., 2010).

In the absence of ovotoxicity, most primordial follicles are naturally degenerated during the apoptotic process of atresia (Mayer et al., 2004). During this process, oocytes are eliminated via apoptosis, and the theca interna cells of atretic follicles become interstitial cells (Hoyer and Sipes, 1996; Hu et al., 2001b; Mayer et al., 2004). These interstitial cells are characteristic of the postmenopausal ovary and produce androgens, such as androstenedione (Mayer et al., 2004). As the mature, antral follicle population is depleted through natural cycling, ovarian failure follows increasing periods of acyclicity (Hoyer and Sipes, 2007). Through VCD-induced elimination of the primordial and small primary follicle populations, the AOF model replicates this cycle of events mimicking human menopause in both acyclicity and final hormone levels (Hoyer and Sipes, 1996, 2007; Lohff et al., 2005; Mayer et al., 2004). Administered at low doses (e.g., 160 mg/kg/day IP in mice; 5 times per week; 15 days total) VCD specifically causes apoptotic cell death of primordial and primary follicles but does not affect other peripheral tissues, including the liver and spleen (for reviews see Borman et al., 1999; Hoyer and Sipes, 1996, 2007; Hu et al., 2001b; Lohff et al., 2005; Muhammad et al., 2009).

This AOF animal model of menopause has several advantages over the intact aging and OVX models. First, in the AOF model animals are intact, not genetically altered, and do not require surgical manipulation. In addition to reducing confounds associated with genetic and surgical manipulations, maintaining the presence of ovarian tissue is an important parallel to transitional menopause in humans. Several studies have recently shown that residual ovarian tissue is steroidogenic, and may contribute to pathologies associated with menopause (Mayer et al., 2004; Rivera et al., 2009). Second, the estrous cyclicity and fluctuations in estrogen, LH and FSH levels in the AOF model are similar to levels in human menopause (Mayer et al., 2004; Mayer et al., 2005). Third, while middle-aged or older animals can be treated to model the menopausal transition in an aged hormonal milieu, VCD injections can be initiated in young animals thus dissociating the effects of altered fluctuations in hormone levels from the effects of aging. Specifically, the mice are injected with VCD as young adults and reach postmenopause 127 days later when they are still young adults. Fourth, longitudinal studies can be performed, and pre-, peri-, and post-menopausal phases can be calculated from set time-points after VCD injections (Fig. 1). By 58 days following the VCD-injection period, mice are in perimenopause and exhibit persistent diestrus (Mayer et al., 2004). By 127 days post VCD-injection period, mice show postmenopausal characteristics similar to humans (Mayer et al., 2004). In particular, ovarian and uterine weights are reduced, plasma LH and FSH are elevated, and plasma estradiol (E2) is non-existent. Fifth, the procedure can be applied to future studies using transgenic or knock-out mice strains, other disease models, or physiological studies in brain slices, since working with slices from older animals can be problematic (Bullock et al., 2008).

The advantages of the AOF model make it an ideal choice for use in studies of menopause and hormone replacement therapy. The chemical nature of the model, and the degree to which it replicates menopause allow this model to be widely-applied to existing and new model systems.

3. TOXICOLOGY AND INFLAMMATORY RESPONSE

VCD is the diepoxide form of 4-vinylcyclohexene (VCH) (IARC, 1982, for review see Hoyer et al., 2001). VCH is an industrial chemical that is an intermediate in the production of synthetic rubber, flame retardants, and polyolefins (Hoyer et al., 2001; IARC, 1982). VCH can be converted, both *in vivo* and *in vitro*, to one of two monoepoxides or a diepoxide (i.e., VCD) (Devine et al., 2001; IARC, 1982; Smith et al., 1990). Much evidence has shown that the *in vivo* conversion of VCH to any of the epoxide forms represents bioactivation, and that the diepoxide form is the most potent ovotoxic form (Borman et al., 1999; Devine et al.,

2001; Hoyer et al., 2001; IARC, 1982; Smith et al., 1990). VCD specifically targets and causes the loss of small, preantral (i.e., primary and secondary follicles) ovarian follicles in rodents (Cannady et al., 2003). VCD seems to act directly on preantral follicles, since it has a similar effect in both *in vivo* and *in vitro* models (Devine et al., 2002).

For many years VCD has been used to model the menopause transition with minimal toxicological effects. As with any pharmacological agent, the effects on the body depend on the dose, duration of exposure and route of administration (Borman et al., 1999; Devine et al., 2001; Hoyer et al., 2001; IARC, 1982; Smith et al., 1990). Although there are reports detailing the emergence of tumors and other pathological outcomes with vinylchloride compounds, these studies used higher concentrations, longer duration, and different routes of administration than those used in the AOF model of menopause (Devine et al., 2001; Hoyer et al., 2001; National Toxicology Program, 1986, 1989). Specifically, early toxicology studies showed that high doses (up to 800 mg/kg) of 4-vinylcyclohexene (VCH), the precursor of VCD, administered through gavage to rodents for 5 days/week for 2 years can have many complications including increased incidences of ovarian and adrenal gland tumors (National Toxicology Program, 1986). Early toxicology studies also showed that high doses of VCD (up to 924 mg/kg) applied dermally to rodents for periods up to 2 years could result in ovarian tumors (National Toxicology Program, 1989). However, these early toxicology reports also indicated that lower doses of VCD delivered for shorter periods selectively targeted the ovary and did not result in tumors (Muhammad et al., 2009; National Toxicology Program, 1989). Thus, for over 15 years, studies have used low doses of VCD to induce ovarian failure to provide insight into reproductive biology (Danilovich and Ram, 2006; Hoyer and Sipes, 1996). The majority of studies have largely focused on understanding the mechanisms of action of VCD in the ovaries.

Because of the results of the early toxicology reports (mentioned above), studies employing VCD have been careful to investigate the potential negative side effects, including tumors. Of the over 25 studies that have employed low doses of VCD (Table 1), none have reported tumors or lesions in the ovaries or adrenals. Low doses of VCD delivered for short durations (usually 15 days) have been shown to specifically cause apoptotic cell death of primordial follicles in the ovaries (Hu et al., 2001a; Hu et al., 2001b; Hu et al., 2002). Cytochrome isoforms known to bioactivate VCH are highly expressed in the interstitial cells of the rodent ovary, and these isoforms have been shown to be modulated by VCH *in vivo* (Cannady et al., 2003). Unlike the VCD precursor VCH that is known to act in the liver (Cannady et al., 2003; Hoyer and Sipes, 1996), low doses of VCD have not been found to affect cytochrome expression or apoptosis markers in the liver (Cannady et al., 2003; Hu et al., 2001a; Hu et al., 2001b; Hu et al., 2002). Moreover, low doses of VCD do not alter the weights of liver, spleen, or kidney (Haas et al., 2007; Lohff et al., 2005; Lohff et al., 2006; Mayer et al., 2004). Pathological examinations have revealed no abnormalities of the liver, kidney, spleen or adrenals in rodents injected with low doses of VCD for short durations and examined up to 240 days post injection (Appt et al., 2006; Golub et al., 2008; Mayer et al., 2004; Mayer et al., 2005; Wright et al., 2008). Studies of the effect of VCD on hepatic levels of glutathione (GSH), an intracellular antioxidant, suggest that treatment with VCD does not cause any hepatic oxidative stress (Devine et al., 2001). At doses 5 times higher than the dose used in the menopause model, VCD has been shown to deplete hepatic GSH levels (Devine et al., 2001). Although hepatic GSH was decreased, at model-levels and exposure, it returned to control levels 26 hours after the last treatment, and ovarian GSH was not affected at any time point (Devine et al., 2001). This is consistent with a role for GSH mediating toxicity of VCD and provides a mechanism by which hepatic GSH can detoxify VCD, but there is no evidence of oxidative stress due to VCD treatment (Mayer et al., 2005). Because preantral follicles are immature, they are not dependent regulation by

gonadotropins, and thus the effect of VCD is not mediated by the hypothalamic-pituitary axis (Hoyer and Sipes, 2007).

Studies in our lab have shown that both acute (1 day post 160 mg/kg, I.P. VCD) and chronic (15 days post 160 mg/kg/day, I.P. @ 1x per day) treatment with VCD fail to induce neuroinflammation (unpublished observations). As a positive control, VCD and vehicle naïve mice were injected with saline or lipopolysaccharide (LPS), which is known to induce a neuroinflammatory response. In LPS-injected mice compared to controls, immunoreactivity to Iba-1, a marker of activated microglia, was visible in processes that were thickened and intensely stained throughout the brain (unpublished observations, Chung et al., 2010; Frank et al., 2007). However, thickened Iba-1-labeled processes were not seen in either the acute or chronic VCD-injected mice compared to control vehicle-injected groups (unpublished observations). These results support the notion that VCD does not have a central inflammatory effect.

4. APPLICATIONS OF THE MODEL

The AOF model of menopause is considered a valid model of menopause (Danilovich and Ram, 2006; Lohff et al., 2005). Unlike the ovariectomy and natural aging models of menopause, the AOF model has great potential for studying the menopause transition period (Williams, 2005). The AOF model has been used primarily to study cardiovascular and metabolic diseases associated with menopause. More recently, the AOF model has been used to study brain disorders impacted by menopause. Below we summarize studies that have utilized the AOF model of menopause.

4.1 Excitotoxic brain damage and neuroprotection

Much of the previous research indicating that estradiol can be neuroprotective for excitotoxic insult to the hippocampus has utilized the OVX model (Schauwecker et al., 2009). Schauwecker et al. (2009) used the VCD model to determine the effect of gradual ovarian hormone loss on the susceptibility to and cell death resulting from a kainate model of epilepsy. They found that mice undergoing OVX- or VCD-induced ovarian failure showed decreased excitotoxic cell death compared to sham- or vehicle-treated mice, in agreement with previous OVX model studies (Schauwecker et al., 2009). Estrogen replacement in OVX animals decreased kainate-induced cell death, but increased seizure duration (Schauwecker et al., 2009). This finding is consistent with previous studies finding cytoprotective effects of estrogen replacement prior to seizure induction (Schauwecker et al., 2009). Future studies aim to determine the effect of estrogen replacement in AOF animals on response to excitotoxic brain injury. The AOF model also will allow for studies of the effect other changes in the menopausal milieu (e.g., androstenedione) on cell death.

4.2 Function of ovarian tissue postmenopause on cognition

As mentioned previously, the shift in ovarian cellular composition from granulosa cells and oocytes to interstitial and stromal cell tissue may contribute to postmenopausal pathologies in women (Mayer et al., 2004). Mayer et al. (2004) used the AOF model to determine the steroidogenic contribution of residual ovarian tissue. AOF ovarian tissue was found to be rich in interstitial and stromal cells. Androstenedione levels persisted in AOF mice after ovarian failure, and follicle-deplete cells produced androstenedione in culture (Mayer et al., 2004). The endocrine and phenotypic characteristics of AOF ovaries resemble that of the postmenopausal milieu (Mayer et al., 2004). These findings were confirmed in an age-matched study, which also found enrichment of mRNA encoding steroidogenic genes (Rivera et al., 2009).

Two studies of the effect of hormone replacement therapy on cognition in rats have found that poor performance on spatial memory tasks correlates with increased levels of androstenedione (Acosta et al., 2009; Acosta et al., 2010). Acosta et al. (2009) directly compared the AOF and OVX models to determine the effect of menopause etiology on cognition as measured by a battery of spatial working memory tasks. Rats were divided into 4 groups: Sham, OVX, AOF, and AOF+OVX. The latter group was OVXed 55 days after beginning VCD treatment (perimenopause) (Acosta et al., 2009). VCD and OVX treatment resulted in poor performance on the behavioral assessments (Acosta et al., 2009). Interestingly, the AOF+OVX group showed improved performance even over the Sham group on some tasks (Acosta et al., 2009). Acosta et al. (2009) found that higher serum levels of androstenedione were associated with decreased performance on the working memory tasks. This association of poor performance with androstenedione is perhaps why the AOF+OVX group outperformed the AOF and OVX groups (Acosta et al., 2009).

Androstenedione was again associated with poor working memory performance in the context of hormone replacement. Acosta et al. (2010) directly compared the effect conjugated equine estrogen (CEE) on OVX and AOF animals. Spatial memory was improved in OVX+CEE animals, but remained poor in the AOF group (Acosta et al., 2010). Androstenedione levels in AOF and AOF+CEE groups were significantly higher than that of the OVX and OVX+CEE groups (Acosta et al., 2010). Increasing androstenedione serum concentration was associated with increased working memory errors (Acosta et al., 2010). While other studies have shown that hormone replacement is effective when administered during perimenopause, in this study CEE was administered after perimenopause (Henderson et al., 2005; Maki, 2006; Zandi et al., 2002). This may account for why the AOF group did not respond to CEE. Nonetheless, the results of this study suggest that menopause etiology and the hormonal milieu that results may have a profound effect on the efficacy of hormone replacement.

Taken together these studies suggest an increasingly important role of androstenedione on menopausal pathologies and response to hormone replacement therapy. The role of residual ovarian tissue in menopause has not been possible to study prior to the AOF model. Continuing work on the role of residual ovarian tissue and associated steroids will be crucial in understanding the effects of menopause and potential treatments.

4.3 Cardiovascular Disease

Since the Women's Health Initiative study contradicted previous reports of the protective effects of ovarian hormones against cardiovascular disease there has been great controversy over the incidence of cardiovascular disease in postmenopausal women and the proper role of hormone replacement therapy (Mayer et al., 2005). Using the AOF model of menopause, Mayer et al. (2005) compared the effect of estradiol replacement on atherosclerotic lesions of AOF, OVXed, and ovary-intact mice. Lipoprotein receptor (LDLR) $-/-$ mice received either VCD or OVX treatment with estradiol replacement, and were then maintained on an high-fat, high-cholesterol diet (Mayer et al., 2005). Mayer et al. (2005) found that AOF and OVX mice that received estradiol supplementation were protected from lesion development and that it resulted in a greater lesion reduction in AOF mice than in OVX mice.

These findings are consistent with other studies in non-human primates that found a protective effect of estradiol replacement prior to cardiovascular insult (Mayer et al., 2005). However, estradiol replacement following a cardiovascular insult has been found to be harmful. This parallels findings in the excitotoxicity literature (Schauwecker et al., 2009). The AOF model will allow for greater study of the effect of estrogen replacement in a menopausal model of cardiovascular disease.

4.4 Metabolic Syndrome and Diabetic Kidney Disease

The AOF model has been used to model the proposed association of menopause and metabolic syndrome and diabetic kidney disease (Keck et al., 2007; Romero-Aleshire et al., 2009). To model metabolic syndrome, both control and VCD-treated mice were fed a high-fat diet (Romero-Aleshire et al. 2009). Romero-Aleshire et al. (2009) found that AOF mice on the high-fat diet demonstrated greater weight gain, higher circulating insulin levels, and had increased insulin resistance relative to normal, cycling mice on the high-fat diet. Even on a standard chow diet, AOF mice demonstrated elevated free fatty acids and cholesterol levels compared to normal, cycling mice (Romero-Aleshire et al., 2009). E2 administration to AOF mice on the high fat diet improved glucose tolerance (Romero-Aleshire et al., 2009). The results of this study suggest that ovarian failure may advance metabolic syndrome, and suggests a role for estrogen replacement in blocking its development (Romero-Aleshire et al., 2009).

The loss of ovarian hormones also is suspected to accelerate diabetic kidney disease (Keck et al., 2007). Keck et al. (2007) treated mice with streptozotocin (STZ) to induce a diabetic state during either perimenopause, 3 days after the end of VCD administration, or 2 weeks post-ovarian failure, 2 weeks after ovarian failure. Induction of diabetes post-ovarian failure resulted in higher blood glucose levels relative to perimenopausal diabetic induction (Keck et al., 2007). STZ treatment post-ovarian failure similarly resulted in a faster onset of renal failure and a change in renal gene expression relative to induction during perimenopause (Keck et al., 2007). This study provides a model for the impact of the menopause transition on kidney disease in diabetes.

The findings of these studies in the AOF model parallel those in the human menopause literature (Keck et al., 2007; Mayer et al., 2004). Given the increasing incidence of diabetes, the AOF model will facilitate our understanding of how changes during menopause will affect insulin resistance and other aspects of metabolic syndrome.

4.5 Carcinogens and Cancer

Postmenopausal incidence of ovarian cancer is 10-fold higher than at premenopausal time points (Hoyer et al., 2009). This increase is thought to be due to loss of oocytes and estrogen production and increased gonadotropic hormone levels (Chakraborty and Gore, 2004; Hoyer et al., 2009; Maffucci and Gore, 2006). The AOF model has been used with an induced ovarian cancer model to demonstrate increased neoplasms, or abnormal cell growths, in follicle depleted mice and rats (Craig et al., 2010; Hoyer et al., 2009). Craig et al. (2010) and Hoyer et al. (2009) used an identical experimental timeline in which animals were treated with VCD or vehicle and subsequently 7,12-dimethylbenz[a]anthracene (DMBA) was injected unilaterally under the ovarian bursa. Tissue was collected 3 and 5 months after the DMBA injections (Craig et al., 2010; Hoyer et al., 2009). No tumors were observed in the absence of VCD treatment regardless of DMBA or oil administration (Craig et al., 2010; Hoyer et al., 2009). All though the mechanism is not known, at both the 3 and 5 month time points, VCD-treated animals had a greater incidence of neoplasms (Hoyer et al., 2009).

Studies of ovarian cancer in rats and mice necessitate intact ovaries and sufficient time after follicle depletion to induce neoplasms. Thus, for this research, the only viable model is the AOF model. However, because it is the only model for this particular application, it cannot be definitively determined that the increase in neoplasm incidence in AOF mice is due only to the administration of DMBA (Craig et al., 2010; Hoyer et al., 2009). Future studies using manipulations of LH, FSH, E2, and androstenedione, will help to clarify increased tumor induction in AOF animals following DMBA administration.

4.6 Fertility

Determining an accurate antral follicle count has been very difficult, and there are few reliable hormonal biomarkers to determine the number of remaining follicles (Sahambi et al., 2008). Anti-Müllerian hormone (AMH) is produced by granulosa cells of small antral primary and secondary follicles in adult ovaries (Mark-Kappeler et al., 2010; Sahambi et al., 2008). However, decreasing levels of serum AMH has been successfully correlated with declining antral follicle count in mice (Sahambi et al., 2008). Existing models of reproductive failure, such as the OVX and intact aging model are not capable of making a detailed assessment of how AMH can be used to accurately assess remaining follicles. Sahambi et al. (2008) chose the AOF model to assess the relationship between AMH and antral follicle numbers. Since VCD eliminates the primordial and primary follicle population, prior to ovarian failure, only antral follicles remain (see section 2.3). AMH was found to correlate with decreasing follicle counts in VCD-treated animals, and in vehicle-treated mice, serum AMH levels correlated with both small and growing follicles (Sahambi et al., 2008). Sahambi et al. (2008) concluded that circulating AMH may be predictive of primordial follicle number. Mark-Kappeler et al. (2010) recently demonstrated that although AMH does not prevent VCD-induced ovotoxicity, it may prevent primordial follicle recruitment to the primary follicle pool by maintaining primordial follicles in a dormant state. Future studies will expand on the developmental role in AMH, as well as its role in fertility, to confirm how ovaries adjust to partial losses of follicle, as is the case with occupational, medical, or environmental exposure to ovotoxicity. The application of VCD-induced ovarian failure will further our understanding of AMH as a biomarker for remaining ovarian follicles and fertility.

4.7 Postmenopausal bone loss

The rapid phase of bone loss and deterioration following menopause in women is thought to be due to a combination of increased bone turnover, and osteoclast-mediated reabsorption (Wright et al., 2008). As with other disease models, the effect of the menopause transition and remaining ovarian tissue on postmenopausal murine skeletons is not possible with traditional menopausal models. AOF animals demonstrated much slower bone loss than their OVX counterparts (Wright et al., 2008). Although both OVX and AOF groups showed deterioration of bone microarchitecture following ovarian loss or failure, the OVX animals demonstrated a trend for greater declines (Wright et al., 2008). There was no effect of VCD treatment on bone morphology or microarchitecture, suggesting that the bone loss and microarchitecture effects are due to ovarian failure, not the VCD treatment (Wright et al., 2008). As in previous studies, Wright et al. (2008) found that OVX animals had lower levels of androstenedione than either AOF or control animals (Mayer et al., 2005; Wright et al., 2008). The authors suggest that the presence of androstenedione may be responsible for slower bone loss in the AOF animals (Wright et al., 2008). Other studies in the lab, suggest that estrogen replacement may reverse ovarian-failure induced bone deterioration (Wright et al., 2008). In this application, the AOF model allows for an unprecedented examination of the effects of estrogen and other menopause-associated hormonal changes absent the confound of age.

4.8 Use in other model species

Since the AOF model is chemical, it can be used in a range of species from rodents to non-human primates. Appt et al. (2006) demonstrated that the AOF model could be successfully adapted to use in non-human primates. The primate ovariectomy model of menopause, like that in rodents, suffers from significant differences with natural menopause, including post-ovarian failure levels of steroid hormones (Appt et al., 2006).

Four female cynomolgus monkeys were treated with VCD (80, 160, or 240 mg/kg) for 15 days. During treatment with VCD, monkeys were assessed for general health. All health measures were found to be in the normal range (Appt et al., 2006). Liver enzymes and injection site inflammation were observed during treatment, but resolved by the end of the injections (Appt et al., 2006). There was no evidence of a toxic effect of VCD treatment at the time of necropsy (Appt et al., 2006). Within the 15 day period, the 160 and 240 mg/kg doses resulted in 50% and 100% loss of primary follicles, respectively (Appt et al., 2006). Further studies will need to determine optimal time course and dosing.

5. SUMMARY

The AOF model of menopause already has been used to study peri- and post-menopausal changes associated with cardiovascular disease (Mayer et al., 2005), diabetes and diabetic kidney damage (Keck et al., 2007), bone loss (Wright et al., 2008), insulin resistance and metabolic syndrome (Romero-Aleshire et al., 2009), fertility (Sahambi et al., 2008) as well as neoplasm development following injection of a known carcinogen into the ovaries (Craig et al., 2010; Hoyer et al., 2009). Additionally, the AOF model of menopause has been used to examine cognitive changes in perimenopause (Acosta et al., 2009; Acosta et al., 2010) and the consequences of ovarian hormone replacement in menopausal Alzheimer's models (Golub et al., 2008) and following excitotoxic brain damage in menopausal mice (Schauwecker et al., 2009). The utility of the VCD model particularly in cardiovascular research has been highlighted in a commentary in a major cardiovascular journal (Williams, 2005). The validity of the model is further exemplified by the fact that Jackson labs (jaxservices@jax.org), after careful studies by their Animal Husbandry and Performance Group, is committed to supplying the "JAX VCD-induced model of menopause." Additionally, the AOF model of menopause has been validated for non-human primates (Appt et al., 2006). As work with this model progresses, it is sure to continue to improve our understanding of the role of estrogen and how its disruption during peri- and postmenopause affects central and peripheral systems. Furthermore, comparing the results of studies in AOF and OVX models will help delineate differences between transitional and surgical menopause in women.

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ABBREVIATIONS

AOF	accelerated ovarian failure
AMH	anti-Müllerian hormone
CEE	conjugated equine estrogen
DMBA	7,12-dimethylbenz[a]anthracene
E2	17 β -estradiol
FSH	follicle stimulating hormone
GnRH	gonadotrophin-releasing hormone
GSH	glutathione
LDLR	lipoprotein receptor
LH	luteinizing hormone

LPS	Lipopolysaccharide
OVX	ovariectomy
STZ	streptozotocin
VCD	4-vinylcyclohexene diepoxide
VCH	4-vinylcyclohexene

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Figure 1. Timeline of AOF onset and estrogen levels and sensitivity
Menopause stage can be calculated in days following initiation of the VCD treatment. (A) Days following the start of the VCD injection period are indicated above the line, while the chronological age of animals is shown below the line. Pre-, peri- and post-menopausal stages are denoted by boxes.

Table 1

Studies of Accelerated Ovarian Failure Summary of doses of VCD and techniques used and organs evaluated of studies investigating or applying the AOF model

Authors	Species and Age	VCD Dose	Time-points	Assessments	Hormones	Organs	Topic
AOF as a Model of Menopause							
Devine et al., 2002	Fischer 344 rats (PND 4-19)	80mg/kg; 0.57 mmol/kg	15d	Follicle counts Histology			In vitro ovarian culture system
Haas et al., 2007	C57BL/6 mice 28d old	160mg/kg IP 15-22d	180d	Follicle counts		Kidney, liver, spleen, adrenals, uterus	Effect of VCD on fertility
Hu et al., 2001a	Fischer 344 rats 28d old	80mg/kg, IP	1d or 15d	Caspase expression and activation		Liver	Ovarian apoptosis in AOF
Hu et al., 2001b	Fischer 344 rats 28d old	80mg/kg IP	1d or 15d	Bcl-2 family member expression		Liver	Change in expression/distribution of Bcl-2 proteins in AOF
Hu et al., 2002	Fischer 344 rats 28d old	80mg/kg IP	1d, 10d or 15d	Expression and binding		Liver	Effect of VCD on ovarian MAPK and AP-1
Keating et al., 2009	Fischer 344 rats In vitro PND4	30uM	2-12d	PI3 kinase signaling			Effect of VCD and DMBA on ovarian PI3
Keating et al., 2010	Fischer 344 rats In vitro PND4	30uM	2-8d	GST and JNK			Effect of VCD on GST
Keating et al., 2010	Fischer 344 rats In vitro PND 4	30uM	2-6d	KIT, AKT, FOXO3			Effect of VCD on PIK3 signaling pathway
Lohiff et al., 2005	C57BL/6 mice 28 d old	160 mg/kg IP 15d	Up to 156d	Cycle length Hormone levels	FSH, E, P	Adrenals, liver, spleen, uterus	Cyclicity and endocrine profile in AOF
Mark-Kappeler et al., 2010	Fischer 344 rats In vitro PND4	30uM	2-8d	AMH mRNA and protein levels			Effect of VCD on AMH distribution and response
Muhammad et al., 2009	Sprague Dawley rats 28d, adult	80, 160 mg/kg 30d		Histology Dosing Body weight		Kidney, liver, lungs, brain	Evaluation of AOF as a model of menopause
Sobinoff et al., 2010	Swiss mice PND 4 In vitro PND 3-4	40, 80 mg/kg 25 uM	PND 4 4d	Microarray, PI3K, AKT, Caspase activity, peroxidation			Comparison of ovotoxic mechanism of VCD, MXC, and MEN
Thompson et al., 2002	Fischer 344 rats 28d old	80 mg/kg IP 15d	15d	Follicle counts Caspase activity	E		Estradiol protection against VCD ovotoxicity
Toxicological Evaluation of AOF							

Authors	Species and Age	VCD Dose	Time-points	Assessments	Hormones	Organs	Topic
Borman et al., 2000	B6C3F mice Fischer 344 rats 28 d old; 15d	80mg/kg IP (+VCH)	15d	Histology Ovotoxic index		Liver	Comparative ovotoxicity of polycyclic aromatic hydrocarbons