REPRODUCTIVE TOXICOLOGY

Effects of chemical mixtures on the ovary

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Abstract

The ovaries play a critical role in female reproductive health because they are the site of oocyte maturation and sex steroid hormone production. The unique cellular processes that take place within the ovary make it a susceptible target for chemical mixtures. Herein, we review the available data regarding the effects of chemical mixtures on the ovary, focusing on development, folliculogenesis, and steroidogenesis. The chemical mixtures discussed include those to which women are exposed to environmentally, occupationally, and medically. Following a brief introduction to chemical mixture components, we describe the effects of chemical mixtures on ovarian development, folliculogenesis, and steroidogenesis. Further, we discuss the effects of chemical mixtures on corpora lutea and transgenerational outcomes. Identifying the effects of chemical mixtures on the ovaries is paramount to preventing and treating mixture-inducing toxicity of the ovary that has long-term consequences such as infertility and ovarian disease.

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Introduction

The ovary is a complex heterogenous organ that is critical for reproductive and systemic health of women. The functional structures within the ovary are follicles at various stages of maturity as well as corpora lutea (CLs) (Guraya 1977). Follicles and CLs are held together by stromal tissue and are surrounded by a layer of germinal epithelium. Each mature follicle contains an oocyte, which is surrounded by many layers of hormone-secreting cells. These cells primarily secrete estradiol and testosterone (Guraya 1977). At its final stage of maturity, a follicle will ovulate, and the oocyte will be released into the oviduct. In response, the remaining cells undergo physical and chemical remodeling to form CLs. The primary function of a CL is to secrete progesterone, which moderates the onset and maintenance of pregnancy. If the oocyte is not fertilized and pregnancy does not occur, the CL regresses and the process of follicle maturation and ovulation begins again (Guraya 1977). The hormones secreted by follicles and CLs work in intricate feedback loops with gonadotropins and hypothalamic hormones in the hypothalamic–pituitary–gonadal axis (Kaprara & Huhtaniemi 2018). The structural diversity and unique biochemical processes that take place in the ovary provide many possible targets for chemical toxicants including pharmaceutical, occupational, and environmental agents.

At the present time, toxicological studies, as well as government exposure regulations, are primarily focused on individual chemicals. As such, a large gap in knowledge regarding the health effects of chemical mixtures exists in general, especially regarding the effects of mixtures on the ovary. Mixtures of chemicals are traditionally thought to act in one of three ways: (1) similar action of chemicals in the mixture, (2) dissimilar action of chemicals in the mixture, or (3) interactions of chemicals in the mixture (synergism or antagonism) (World Health Organization 2017). The assumption is made that chemicals that have similar or dissimilar actions do not interact and the toxicological profiles of each individual chemical remain unchanged in the mixture. The consensus in the United States (US) and the European Union (EU) is often that a mixture of chemicals can be assessed based on an assumption of additivity (World Health Organization 2017). That is, a mixture is deemed safe if all the individual chemical components are present at concentrations below the no observable adverse effect level (NOAL). However, epidemiological data, animal studies, and mechanistic data all support the notion that chemical interactions are far more complex and as such, chemicals must be studied in combination, regardless of their individual toxicological profiles. In this review, we discuss the effects of chemical mixtures on the ovary, with an emphasis on ovarian development, folliculogenesis, and

steroidogenesis. These data collected from *in vitro* and *in vivo* experiments can be integrated to understand the mechanisms through which various chemical mixtures cause disruption in the ovarian processes within the whole animal.

Chemical mixture components

The chemical mixtures discussed in this review include agricultural chemicals, plasticizers, flame retardants, phytoestrogens, pharmaceuticals, cosmetic agents, and industrial by-products. Although some of the mixtures are composed of a single class of chemicals, others represent combinations of various classes of chemicals. Many of these chemicals have been individually identified as potent ovarian toxicants and their combinations in mixtures pose a unique threat to female reproductive health. Pesticides are primarily used in agricultural industries for large-scale crop production; however, they are also used in veterinary medicine as flea and tick preventatives for pets, and in human medicine as antifungal drugs. Pesticides accumulate in the environment and are often detected in house dust as well as on produce (Sciences 2020). Phthalates are ubiquitous industrial chemicals used as plasticizers and solvents in building materials, cosmetics, food storage containers, medical equipment, and children's toys (Zhou et al. 2017a). The mixtures discussed in this review are composed of both parent phthalate compounds as well as their monoester metabolites. Another class of plasticizers found in mixtures of ovarian toxicants are bisphenols. Bisphenols are plasticizers used in the manufacturing of polycarbonate plastics and epoxy resins. They are found in food storage containers, thermal receipt papers, medical equipment, and industrial piping (Ben-Jonathan & Hugo 2016). Bisphenol A (BPA) has been studied extensively as a reproductive toxicant and it is found in the mixtures discussed in this review. Flame retardants are synthetic chemicals used in various industries to delay or prevent rapid thermal acceleration and fires. Commonly used classes of flame retardants include polychlorinated biphenyls (PCBs) and brominated flame retardants (BDEs). PCBs are synthetic chemicals used as coolants and lubricants in electrical equipment. Although PCBs are no longer produced in the US, they accumulate in the environment and do not break down easily (Agency 2020). As a result, women continue to be exposed to PCBs long after their removal from industrial markets in the US. Phytoestrogens are plant-derived chemicals that resemble estrogen in structure. Women are largely exposed to phytoestrogens through dietary supplements and consumption of phytoestrogen-rich foods like soy (Patisaul & Jefferson 2010). The phytoestrogens discussed in this review include flavones and isoflavones. Pharmaceuticals are drugs synthesized for treating, curing, or preventing diseases. Although pharmaceutical drugs are intended

to be beneficial therapeutics, they also can have adverse reproductive outcomes (Schmitz et al. 2018). Over the counter drugs and drugs used for the treatment of chronic conditions are of concern due to their extended period of use. Parabens are used as preservatives and anti-microbials in food products and cosmetics (Zhou et al. 2017a). Women are exposed to parabens by consumption of contaminated food products and the use of cosmetics. Chemical UV filters are another class of toxicants found in cosmetics. They are most commonly used in sunscreens, hairsprays, and other cosmetics to prevent damage from sun exposure (Zhou et al. 2017a). Women are exposed to chemical UV filters when they use sunscreens, hairsprays, and other cosmetics. Ovarian toxicants found in cosmetics and personal care products are of concern because data show that women use more of these products at a higher frequency than men (Zhou et al. 2017a). Polycyclic aromatic hydrocarbons (PAHs) are by-products of the incomplete combustion of organic materials including coal, gas, oil, and wood. According to the Centers for Disease Control (CDC), women are exposed to PAHs by inhaling contaminated air from cigarettes and vehicle exhausts as well as by consuming grilled or charred foods (Prevention 2009). Wastewater from hydraulic fracturing is another major source of exposure to industrial by-products. It is often highly contaminated with both organic and inorganic chemicals (Kassotis et al. 2016). Refer to Table 1 for a list of the individual chemicals found in the mixtures discussed in this review.

Effects of chemical mixtures on ovarian development

Through several complex processes, the ovaries of a developing fetus begin to form shortly after sex determination. Ovarian development begins with extensive proliferation of both primordial germ cells and somatic cells in clusters termed germ cell nests (Hirshfield 1991). Eventually, the somatic cells reorganize themselves to form a single layer around a germ cell nest and subsequently, the germ cell nests stop dividing and the cells in the nests undergo meiosis to form oocytes. After approximately 70% of oocytes in the germ cell nest undergo apoptosis, the nest breaks down and the somatic cells rearrange themselves to form a single layer around each surviving oocyte (Hirshfield 1991). This structure, which is complete with an individual oocyte surrounded by a single layer of granulosa cells, is called the primordial follicle. Females are born with a finite pool of resting primordial follicles (Guraya 1977). Throughout a female's reproductive lifespan, these follicles grow into more mature follicles capable of ovulation and production of sex steroid hormones. Healthy adult ovaries contain a diverse follicle pool including primordial, primary, secondary, and mature antral follicles (Hirshfield 1991). Exposures to chemical

Table 1 Chemical mixture components.

	Description	Exposure	Chemicals	References
Pesticides				
Insecticides	Used in agricultural industries for large scale crop production; however, they are also used in veterinary medicine as flea and tick preventatives for pets	Inhalation of house dust, consumption of contaminated foods	DDT, DDD, DDE, MXC, DEET, Permethrin aroclor, chlordane, taxophene, aldrin, dieldrin, 1,2,4,5-tetrachlorobenzene, α -HCH, β -HCH, hexachlorobenzene, mirex, lindane, pentachlorobenzene	Baldrige 2003, Rice <i>et al.</i> 2006, Prevention 2009, Wang <i>et al.</i> 2013, Vannuccini <i>et al.</i> 2016, Lea <i>et al.</i> 2016, Kaprara & Huhtaniemi 2018, Pollock <i>et al.</i> 2018, Johansson <i>et al.</i> 2020, Meling <i>et al.</i> 2020
Fungicides	Used in large scale agricultural industries to as well as in human and veterinary medicine to prevent fungal infestations and treat fungal infections	Consumption of contaminated foods and topical or oral medications	Vinclozolin, prochloraz, procymidone, epoxiconazole	Baldrige 2003, Gregoraszczuk <i>et al.</i> 2005, Lea <i>et al.</i> 2016
Plasticizers				
Phthalates and phthalate metabolites	Used as plasticizers and solvents in cosmetics, food storage containers, medical equipment, and children's toys	Consumption of contaminated food products and environmental exposure from bisphenols leaching out of products	DEP, DEHP, DBP, DiBP, DiNP, BBzP, MEP, MEHP, MBP, MiBP, MiNP, MBzP	Guraya 1977, Hirshfield 1991, Gregoraszczuk <i>et al.</i> 2005, Bellingham <i>et al.</i> 2013, Wang <i>et al.</i> 2013, 2014, Johansson <i>et al.</i> 2016, Lea <i>et al.</i> 2016, Zhou <i>et al.</i> 2017a, Hernández-Ochoa <i>et al.</i> 2018, Samardzija Nenadov <i>et al.</i> 2018
Bisphenols	Used in the manufacturing of polycarbonate plastics and epoxy resins. They are found in food storage containers, thermal receipt papers, medical equipment, and industrial piping.	Consumption of contaminated food products and environmental exposure from bisphenols leaching out of products	BPA	Gregoraszczuk <i>et al.</i> 2005, Wang <i>et al.</i> 2013, Ben-Jonathan & Hugo 2016, Lea <i>et al.</i> 2016, Zhou <i>et al.</i> 2017a, Samardzija Nenadov <i>et al.</i> 2018
Flame retardants				
PCBs	Used as coolants and solvents in electrical equipment, rubber products, and paints	Consumption of contaminated fish and environmental exposure from PCBs leaching out of products	PCB: 28, 52, 101, 118, 126, 138, 153, 180	Guraya 1977, Borgert <i>et al.</i> 2004, Prevention 2009, Karpeta & Gregoraszczuk 2010, Patisaul & Jefferson 2010, Kaprara & Huhtaniemi 2018, Pollock <i>et al.</i> 2018
Brominated flame retardants	Used as coolants in electronics, furniture, and textiles	Inhalation of dust	PBDE: 28, 47, 99, 100, 153, 154, 183, 209; DE: 71, 79, decaBDE209; HBCD, TBBPA	Guraya 1977, Rice <i>et al.</i> 2006, Prevention 2009, Kassotis <i>et al.</i> 2016, World Health Organization 2017, Kaprara & Huhtaniemi 2018, Pollock <i>et al.</i> 2018
Phytoestrogens				
Isoflavones	Plant-derived estrogens found in legumes, especially soybeans and clover	Consumption of soy-based food products like tofu and soymilk as well as over the counter dietary supplements	Genistein, daidzein, biochanin A, glycerin	Campagna <i>et al.</i> 2001, Kraugerud <i>et al.</i> 2012, Schmitz <i>et al.</i> 2018, Sciences 2020
Flavones	Plant-derived estrogens found in red and yellow fruits and vegetables	Consumption of red and yellow fruits and vegetables as well as over the counter dietary supplements	Apigenin, quercetin	Campagna <i>et al.</i> 2001, Schmitz <i>et al.</i> 2018

(Continued)

Table 1 Continued.

	Description	Exposure	Chemicals	References
Pharmaceuticals				
Non-steroidal drugs	Drugs synthesized for treating, curing, or preventing diseases	Over the counter or prescription use	Paracetamol, diclofenac, naproxen, triclosan, carbamazepine, irbesartan	Gregoraszczuk <i>et al.</i> 2005, Fowler <i>et al.</i> 2008, Lea <i>et al.</i> 2016, Samardzija Nenadov <i>et al.</i> 2018
Cosmetics				
Parabens	Used as preservatives and anti-microbials in food products and cosmetics	Consumption of contaminated food products and use of cosmetics	Butyl paraben, propyl paraben	Gregoraszczuk <i>et al.</i> 2005, Bellingham <i>et al.</i> 2013, Samardzija Nenadov <i>et al.</i> 2018
UV-filters	Used in sunscreens, hairsprays, and other cosmetics to prevent damage from sun exposure	Use of cosmetics containing UV-filters	4-MBC, OMC	Gregoraszczuk <i>et al.</i> 2005, Bellingham <i>et al.</i> 2013, Lea <i>et al.</i> 2016
Industrial by-products				
Polycyclic aromatic hydrocarbons	By-products of the incomplete combustion of organic materials including coal, gas, oil, and wood	Inhalation of contaminated air from cigarettes and vehicle exhausts as well as consumption of grilled or charred foods	Naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, benzo[ghi]perylene	Guraya 1977, Gregoraszczuk <i>et al.</i> 2008a
Hydraulic fracturing chemicals	The wastewater from hydraulic fracturing processes is highly contaminated with both organic and inorganic chemicals	Environmental exposure to contaminated water	1,2,4-Trimethylbenzene, 2-(2-methoxyethoxy) ethanol, 2-ethylhexanol, 2-methyl-4-isothiazolin-3-one, acrylamide, benzene, bronopol, cumene, diethanolamine, ethoxylated nonylphenol, ethoxylated octylphenol, ethylbenzene, ethylene glycol, ethylene glycol monobutyl ether, naphthalene, N,n-dimethyl-formamide, phenol, propylene glycol, sodium tetraborate decahydrate, styrene, toluene, triethylene glycol, xylenes	Gregoraszczuk <i>et al.</i> 2008b

4-MBC, 4-methylbenzylidenecamphor; BBzP, butyl benzyl phthalate; DBP, dibutyl phthalate; DDD, dichlorodiphenyldichloroethane; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; DEET, N, N-diethyl-meta-toluamide; DEHP, di(2-ethylhexyl) phthalate; DEP, diethyl phthalate; DiBP, diisobutyl phthalate; DiNP, diisononyl phthalate; HBCD, hexabromocyclododecane; MBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEP, monoethyl phthalate; MiBP, monoisobutyl phthalate; MiNP, monoisononyl phthalate; MXC, methoxychlor; OMC, octyl methoxycinnamate; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; TBBPA, tetrabromobisphenol A; α -HCH, α -hexachlorocyclohexane; β -HCH, β -hexachlorocyclohexane.

mixtures during embryonic ovarian development can and have been shown to affect ovarian morphology in a way that has negative consequences for ovarian function. Abnormal ovarian morphology, including weight discrepancies, irregular proportions of follicle populations, atretic follicles, cysts, and fibrosis are all indicative of functional pathology (Hernández-Ochoa *et al.* 2018).

Although no studies have examined the effects of chemical mixtures on germ cell nest formation and

breakdown, studies do show that mixtures can alter gene expression or protein levels in the developing ovary. For example, a mixture of nonsteroidal pharmaceuticals (paracetamol, carbamazepine, diclofenac, irbesartan, and naproxen) increased expression of forkhead box L3 (*Foxl3*), a transcription factor maintained only in female germ cells, during fetal sex determination in juvenile trout (Schmitz *et al.* 2018). A mixture of both antiandrogenic and estrogenic chemicals (DBP, DEHP, BPA, vinclozolin, prochloraz, procymidone, linuron,

epoxiconazole, *p,p'*-DDE, 4-MBC, OMC, paracetamol, and butyl paraben) dysregulated 106 proteins compared to control in the ovaries of Wistar rats exposed during ovarian development. The proteins identified were involved in post-transcriptional RNA processing, cytoskeletal organization, intracellular trafficking, and detoxification (Johansson *et al.* 2020). A mixture of antiandrogenic chemicals (DBP, DEHP, vinclozolin, prochloraz, procymidone, linuron, epoxiconazole and *p,p'*-DDE) dysregulated 19 proteins compared to control in ovaries from developmentally exposed Wistar rats. The dysregulated proteins were involved in nuclear transport, cytoskeletal organization, and metabolism (Johansson *et al.* 2020). A mixture of estrogenic chemicals (4-MBC, OMC, BPA, and butyl paraben) dysregulated 12 proteins compared to control in the ovaries of developmentally exposed Wistar rats. The dysregulated proteins were involved in translation and intracellular signal transduction that are active during ovarian development (Johansson *et al.* 2020). In addition, in utero exposure to a mixture of endocrine disrupting chemicals (DEHP, PCB congeners: 28, 52, 101, 118, 138, 153, and 180, PBDE congeners: 28, 47, 99, 100, 153, 154, 183, and PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, and benzo[ghi]perylene) reduced total oocyte densities and expression of growth differentiation factor (GDF9), an ovarian growth factor involved in oocyte development, growth and function in sheep. (Fowler *et al.* 2008). Further, temporal exposure to the mixture resulted in differential expression of several transcripts in the

ERK/MAPK and PI3K/AKT signaling cascades, which are heavily involved in somatic and germ cell dialogue during early proliferation and migration events (Lea *et al.* 2016).

Effects of chemical mixtures on folliculogenesis

Folliculogenesis is the process through which immature follicles mature to hormone-secreting follicles capable of ovulation (Hirshfield 1991). The process begins with primordial follicles, which contain an oocyte surrounded by one layer of squamous granulosa cells. The single granulosa cell layer transforms into a cuboidal granulosa layer and the follicles are now referred to as primary follicles (Hirshfield 1991). Primary follicles mature to secondary follicles in which the oocyte is surrounded by many layers of granulosa cells. Surrounding these many layers of granulosa cells is a thin layer of squamous theca cells. The follicle, now complete with all its cellular components, continues to grow until it becomes a mature antral follicle (Hirshfield 1991). The antral follicle is complete with an oocyte surrounded by many layers of granulosa cells and outer thecal cell layers. The mature antral follicle also contains an antrum, a fluid filled space that contains steroid hormones, proteins, polysaccharides, and other maintenance and signaling molecules. The antral follicle is the only follicle capable of ovulation and it is equipped with the cellular machinery to provide the ovary and the rest of the body with adequate estradiol and testosterone (Hirshfield 1991). For this reason, it is often referred to as the functional unit of the ovary and it is the subject of many ovarian toxicology studies. Mixtures of chemicals that are toxic to ovarian follicles can impact the number

Figure 1 Schematic of development of the primordial follicle pool and folliculogenesis showing chemical mixtures that affect each follicle stage. Oocytes are pink, granulosa cells are purple, and theca cells are yellow.

of viable oocytes available for fertilization and interfere with the production of sex steroid hormones. Importantly, women are born with a finite number of primordial follicles (Guraya 1977). Ovarian toxicants that interfere with any stage of folliculogenesis might accelerate the rate at which the primordial follicle pool is depleted, leading to infertility due to premature ovarian failure and other ovarian pathologies (Hernández-Ochoa et al. 2018). Refer to Fig. 1 for a schematic of folliculogenesis showing chemical mixtures that affect each follicle stage.

Several studies have shown that mixtures can adversely affect follicle growth and death *in vitro*. A mixture of phthalate metabolites (MEP, MEHP, MBP, MiBP, MiNP, MBzP) inhibited growth of mouse antral follicles compared to control (Melting et al. 2020). This inhibition of follicle growth is likely due to mixture-induced effects on expression of cell cycle regulators and apoptotic factors (Melting et al. 2020). It also could be due to the phthalate metabolite mixture affecting expression of several receptors and receptor associated genes compared to control (Melting et al. 2020). Namely, the mixture dysregulated expression of peroxisome proliferator-activated receptors, the aryl hydrocarbon receptor (*Ahr*), and hormone receptors compared to control (Melting et al. 2020). A similar study showed that a phthalate mixture (DEHP, DEP, DBP, DiBP, BBzP, DiNP) increased fragmented oocytes and reduced expression of antioxidant enzymes compared to control (Zhou & Flaws 2017). A mixture of 10 different organochlorine pesticides (Aroclor, chlordane, *p,p'*-DDT, *p,p'*-DDE, taxophene, α HCH, aldrin, dieldrin, 1,2,4,5-tetrachlorobenzene, *p,p'*-DDD, β HCH, hexachlorobenzene, mirex, lindane, and pentachlorobenzene) reduced cumulus expansion and reduced cumulus cell viability in porcine cumulus oocyte complexes compared to control (Campagna et al. 2001). Collectively, these *in vitro* studies indicate that mixtures can adversely affect follicle health.

Studies have also shown that mixtures can affect folliculogenesis *in vivo*. A mixture of PCBs, PBDEs, and *p,p'*-DDT suppressed ovarian follicle development by reducing follicular proliferation and increasing apoptosis in zebra fish (Kraugerud et al. 2012). In utero and lactational exposure to Aroclor 1016, a commercially prepared PCB mixture, significantly reduced the numbers of preantral and antral follicles as well as increased atresia of antral follicles in the ovaries of exposed rats compared to control (Baldridge 2003). A mixture of PBDEs (DE-71, DE-79, decaBDE-209, and HBCD) increased preantral and antral follicle numbers and enlarged antral follicles compared to control in Sprague Dawley rats (Lefèvre et al. 2016). A mixture of soy isoflavones (genistein, daidzein, and glycerin) decreased the numbers of primordial and primary follicles, increased the numbers of atretic follicles, and increased the number of large antral follicles compared to control in Wistar rats. Further, the follicles of the

exposed rats had higher occurrences of multiple and binuclear oocytes compared to control (Wang et al. 2013). In addition, the follicular fluid of the exposed rats contained less estradiol and progesterone and more cholesterol and cortisol compared to control (Wang et al. 2013). Perinatal exposure to a mixture of phthalates (DBP and DEHP), pesticides (vinclozolin, prochloraz, procymidone, linuron, epoxiconazole, *p,p'*-DDE), UV-filters (4-MBC and OMC), BPA, butylparaben, and paracetamol significantly reduced primordial follicle numbers in rats. The mixture also caused irregular estrous cycles and reduced ovarian weights compared to control when animals reached 1 year of age (Johansson et al. 2016). Prenatal exposure to a mixture of 23 hydraulic fracturing chemicals caused dose dependent disruptions in folliculogenesis, with an overall increase in atretic follicles compared to control in the F1 offspring of C57Bl/6 mice (Kassotis et al. 2016). Another mixture (DEHP, PCB congeners: 28, 52, 101, 118, 138, 153, and 180, PBDE congeners: 28, 47, 99, 100, 153, 154, 183, and PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenzo[a,h]anthracene, and benzo[ghi]perylene) increased cell death in ovarian follicles and increased the numbers of mature follicles in the ovaries of in utero exposed offspring compared to control ewes (Fowler et al. 2008). The mixture also caused differential expression of proteins involved in gene expression/transcription, protein synthesis, phosphorylation, and receptor activity in the ovarian tissues of exposed offspring compared to control in ewes (Fowler et al. 2008). Further, ewes exposed to the same mixture after conception had significantly decreased primordial follicle numbers compared to ewes treated during preconception and compared to control (Bellingham et al. 2013). Interestingly, these changes were observed only in ewes temporarily exposed to the mixture and not observed in the ewes exposed to the mixture continuously throughout gestation (Lea et al. 2016). Collectively, these results show that chemical mixtures adversely affect the process of folliculogenesis as well as follicle health *in vivo*.

Effects of chemical mixtures on steroidogenesis

The antral follicle is the primary hormone secreting unit within the ovary. In response to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary, theca cells and granulosa cells work together to produce the sex steroid hormones estradiol and testosterone (Hirshfield 1991). Cholesterol is transported into the theca cell by steroidogenic acute regulatory (STAR) (STAR) protein. It is then converted to pregnenolone by cholesterol side-chain cleavage enzyme (CYP11A1). Pregnenolone can be converted

to dehydroepiandrosterone by cytochrome P450 17A1 (CYP17A1) or it can be converted to progesterone by 3 β -hydroxysteroid dehydrogenase (HSD3B1). Dehydroepiandrosterone and progesterone can both be converted to androstenedione by the same two enzymes. Androstenedione can remain in the theca cell where it is converted to testosterone by 17 β -hydroxysteroid dehydrogenase (HSD17B) or it can diffuse into the granulosa cell where it is converted to estrone by aromatase (CYP19A1). Estrone is finally converted to estradiol by HSD17B. Testosterone can also diffuse into the granulosa cell where it is terminally converted to estradiol by CYP19A1. Many ovarian toxicants are endocrine disrupting chemicals, which interfere with the ability of the ovary to synthesize and metabolize estradiol and testosterone. Hormonal imbalances resulting from an excess or deficiency in sex steroid hormones is associated with adverse fertility and health outcomes in women (Vannuccini *et al.* 2016). Refer to Fig. 2 for a schematic of steroidogenesis showing mixtures that affect expression of steroidogenic regulators.

Several *in vitro* studies indicate that chemical mixtures can affect ovarian steroidogenesis. A mixture of *p,p'*-DDE and MXC inhibited testosterone production compared to control in cultured female bass gonads (Borgert *et al.* 2004). A mixture of phthalate metabolites (MEP, MEHP, MBP, MiBP, MiNP, MBzP) increased expression of *Star*, but decreased expression of all downstream steroidogenic enzymes compared to control in cultured mouse follicles (Meling *et al.* 2020). Further, the mixture increased the concentration of pregnenolone, progesterone, androstenedione, testosterone, and estradiol in the media at 24 h and it increased the concentrations of DHEA, androstenedione, and testosterone and decreased the concentration of estradiol compared to control at 96 h (Meling *et al.* 2020). The mixture-induced decrease in estradiol corresponds

to upregulation of *Cyp11a1*, an enzyme that metabolizes estradiol (Meling *et al.* 2020). A mixture of estrogenic extracts isolated from untreated wastewater increased expression of the steroidogenic factors *Star* and *Hsd3b1* in cultured mouse granulosa cells (Samardzija Nenadov *et al.* 2018). This effect was likely mediated through activation of estrogen receptors (Samardzija Nenadov *et al.* 2018). In other studies, a PBDE mixture (PBDE 47, 99, 100, and 209) was applied to co-cultured theca and granulosa cells in low (0.5; 0.25; 0.1; 0.05 ng/mL), medium (25; 10; 4.0; 0.25 ng/mL), and high (50; 17.5; 12.5; 0.5 ng/mL) doses in short term and long-term culture periods. In the short-term experiments, low doses of the mixture increased estrogen, testosterone and progesterone secretion compared to control (Karpeta & Gregoraszcuk 2010). In the long-term experiments, medium doses of the mixtures stimulated estradiol and testosterone secretion, and the low, medium, and high dose mixtures stimulated progesterone secretion compared to control (Karpeta & Gregoraszcuk 2010). These effects were irreversible after removal of mixture reagents (Karpeta & Gregoraszcuk 2010). Further, studies show that the commercial PCB mixture Delor 103 increased testosterone secretion after a single exposure and stimulated estradiol secretion after permanent treatment compared to control in cultured porcine theca interna and granulosa cells. The PCB mixture Delor 106 had antiestrogenic effects on the cultured cells after a single exposure and significant estrogenic effects on the permanently treated cells compared to control (Gregoraszcuk *et al.* 2005). In addition, exposure to a mixture of persistent organic pollutants (POPs) (*p,p'*-DDE, PCB-138, PCB-153, *p,p'*-DDD, and CHB 50) decreased concentrations of estradiol and progesterone and increased the concentrations of testosterone in the media of cultured ovarian cells compared to control (Gregoraszcuk *et al.* 2008b).

Figure 2 Schematic of steroidogenesis in ovarian follicular cells showing which mixtures affect expression of steroidogenic regulators. Granulosa cells are depicted in purple and theca cells are depicted in yellow.

Exposure to the same mixture diluted by factors of 2, 4, 20, 40, 200, and 1,000 increased the concentrations of estradiol, progesterone, and testosterone in the cultured ovarian cells compared to control. A different mixture of POPs (BDE-47, BDE-99, HBCD, BDE-100, and *p,p'*-DDE) increased the concentrations of estrogen and testosterone in the media of cultured ovarian follicles compared to control (Gregoraszczyk *et al.* 2008b). In contrast, the mixture decreased the concentration of progesterone in the media of cultured ovarian cells compared to control. Exposure to a third mixture of POPs (*p,p'*-DDE, HBCD, BDE-47, PCB-153, and BDE-99) decreased the concentrations of estradiol and progesterone in the cultured ovarian cells compared to control and increased the concentration of testosterone in the media of cultured ovarian cells compared to control (Gregoraszczyk *et al.* 2008b). Exposure to the same mixture diluted by factors of 2, 4, 20, 40, 200, and 1000 yielded significantly more profound effects than the undiluted mixture (Gregoraszczyk *et al.* 2008b).

A PCB+*p,p'*-DDT mixture (PCB-153, 126, 118, 138, 180, *p,p'*-DDT) decreased estradiol and testosterone in the media of cultured porcine ovarian cells compared to control. When *p,p'*-DDT was replaced with *p,p'*-DDE, the mixture increased estradiol and decreased testosterone levels compared to control in cultured porcine ovarian cells. When both *p,p'*-DDT and *p,p'*-DDE were used in the mixture, the mixture increased the concentration of estradiol and decreased the concentration of testosterone compared to control in the media of cultured porcine ovarian cells (Gregoraszczyk *et al.* 2008c). A mixture of BDEs (BDE-47, BDE-99, BDE-100, BDE-209) increased estradiol and testosterone in the media of cultured porcine ovarian cells compared to control (Gregoraszczyk *et al.* 2008a). The addition of *p,p'*-DDT to the mixture increased the concentrations of testosterone, but did not affect estradiol concentrations compared to control in the media of cultured porcine ovarian cells (Gregoraszczyk *et al.* 2008a). The addition of *p,p'*-DDE to the mixture increased the concentrations of estradiol and testosterone compared to control in cultured porcine ovarian cells. The addition of both *p,p'*-DDT and *p,p'*-DDE to the BDE mixture increased the concentrations of both estradiol and testosterone compared to control in cultured porcine ovarian cells (Gregoraszczyk *et al.* 2008a). Collectively, these studies show that mixtures can affect the production of sex steroid hormones in various ovarian culture systems.

Studies also show that mixtures affect steroidogenesis *in vivo*. Specifically, a mixture of nonsteroidal pharmaceuticals (paracetamol, carbamazepine, diclofenac, irbesartan, and naproxen) significantly increased plasma 11-ketotestosterone and plasma estradiol as well as increased ovarian expression of estrogen receptor beta (*Esr1*), and steroidogenic factors *Cyp17a1*, *Cyp19a1*, and *Hsd11b* in female juvenile trout (Schmitz *et al.* 2018). A mixture of PBDEs (DE-71, DE-79, decaBDE-209, and hexabromocyclododecane)

decreased ovarian expression of *Cyp17a1* as well as circulating levels of 17-hydroxypregnenolone and increased testosterone in rats (Lefèvre *et al.* 2016). Interestingly, exposure to a mixture of triclosan, tetrabromobisphenol A (TBBPA), butyl paraben, propyl paraben, and DEHP led to elevated concentrations of BPA in the ovaries and increased urinary estradiol concentrations compared to control in CF1 mice, suggesting that toxicant mixtures compete for enzymes in BPA and estrogen metabolic pathways (Pollock *et al.* 2018).

Effects of chemical mixtures on corpora lutea

Formation of the CL results from remodeling, growth, and differentiation of theca and granulosa cells in response to a LH surge from the anterior pituitary. Once formed, the primary function of a CL is to synthesize and secrete progesterone, which is necessary for initiation and maintenance of a successful pregnancy. The CL has a short lifespan. If pregnancy does not occur, the CL stops producing progesterone and undergoes regression.

A few studies show that mixtures can affect the function of the CL *in vitro* and *in vivo*. For example, exposure to a mixture of isoflavones (biochanin A, genistein and daidzein) reduced *Cyp19a1* mRNA expression and activity compared to control in cultured granulosa-luteal cells (Rice *et al.* 2006). A mixture of the flavones apigenin and quercetin reduced aromatase activity compared to control in cultured granulosa-luteal cells (Rice *et al.* 2006). A mixture of soy isoflavones (genistein, daidzein, and glycitein) increased the numbers of CLs and the numbers of apoptotic cells in the CLs compared to control in Wistar rats (Wang *et al.* 2013, 2014). Collectively, these studies indicate that mixtures of phytoestrogens adversely affect CLs *in vitro* and *in vivo*.

Transgenerational effects of chemical mixtures on the ovary

Exposures to chemical mixtures during fetal development can cause permanent changes to the ovarian epigenome, resulting in adverse reproductive outcomes later in adult life such as infertility, primary ovarian insufficiency, and polycystic ovarian syndrome (PCOS) (Nilsson *et al.* 2012). Specifically, prenatal exposure to an environmentally relevant phthalate mixture (DEHP, DEP, DBP, DiNP, DiBP, BBzP) increased ovarian cysts compared to control in CD-1 mice (Zhou *et al.* 2017b). Prenatal exposure to either a pesticide mixture (permethrin and DEET) or a plastics mixture (BPA, DEHP, DBP) from gestational day 8 to 14 caused reproductive abnormalities in the F3 generation of Sprague Dawley rats. Specifically, the plastics mixture caused early onset puberty compared to control and both the pesticide and plastics mixtures caused a significant decrease in the primordial follicle pool compared to

control (Manikkam *et al.* 2012). In a similar study with the same mixtures and same dosing paradigm, both the pesticide mixture and the plastics mixture reduced primordial follicle numbers compared to control in the F1 and F3 generations. The plastics mixture also increased the numbers of small and large cysts in the F1 generation compared to control, and both mixtures increased small and large cysts in the F3 generation compared to control (Nilsson *et al.* 2012). Finally, the pesticide mixture reduced large antral follicles in the ovaries of the F3 generation compared to control (Nilsson *et al.* 2012). In another similar study, gestating Sprague Dawley rats were exposed to a mixture of plasticizers (BPA, DEHP, DBP) from gestational day 8 to 14. The exposed F1 generation had decreased ovary weights compared to control rats. Both the F1 and F3 generations had increased pubertal abnormalities compared to control rats, including delayed puberty in the F1 generation and early onset puberty in the F3 generation compared to control. Exposed rats in both F1 and F3 generations had primordial follicle loss, an increased number of cysts, and decreased follicular reserve compared to control. Further, the F3 ovaries contained 197 differential DNA methylation regions (DMRs), many of which correlated to the morphological and pathological changes observed in the ovaries of the F3 generation (Nilsson *et al.* 2012). Collectively, these data show that in utero exposure to a plastics mixture during the period of fetal gonadal sex determination promotes epigenetic transgenerational inheritance of adult onset disease resembling primary ovarian insufficiency and polycystic ovarian syndrome (PCOS).

Conclusion

The diverse cellular composition and unique processes that happen in the ovary make it a susceptible target for chemical mixtures. The data reviewed come from *in vitro* experiments designed to test the effects of chemical mixtures directly on ovarian tissue and *in vivo* experiments which represent ovarian toxicity in the whole reproductive axis. *In vitro* experiments are useful to understand the mechanisms through which chemical mixtures cause ovarian toxicity in animal studies. Together, these data indicate that chemical mixtures can affect ovarian development, folliculogenesis, and steroidogenesis. However, further studies are required to close the remaining gaps in knowledge regarding the effects of chemical mixtures on the ovary. We need to identify the compositions of environmentally relevant mixtures and study how they affect the ovary. Furthermore, mixtures in the US and EU are currently regulated based on the assumption that chemicals in a mixture either have similar or dissimilar actions and as such are assessed based on the toxicological profiles of each individual component in the mixture (World Health Organization 2017). The data described in this review coupled with human

epidemiological data support the notion that chemical mixture interactions are, in fact, more complicated. As such, we need to identify the unique mechanisms of action of chemical mixtures regardless of the mechanisms of their individual chemical components. Through future studies, we can develop a more robust understanding of how mixture components interact with each other and behave differently than they might alone. Using this information on toxicity and mechanisms of action of chemical mixtures, we can develop strategies to prevent or treat mixture-induced toxicity of the ovary.

Declaration of interest

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

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Author contribution statement

V M performed the literature review, wrote the paper, and designed the table and figures. J F revised the paper and figures and approved the final version to be published.

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