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Investigating the effects of chemical exposure, demographic, and lifestyle factors on steroid hormone receptor bioactivity in human populations

By

Phum Tachachartvanich

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in
Environmental Health Sciences

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Martyn T. Smith, Chair
Professor Stephen M. Rappaport
Professor Luoping Zhang
Associate Professor Jen-Chywan Wang

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Abstract

Investigating the effects of chemical exposure, demographic, and lifestyle factors on steroid hormone receptor bioactivity in human populations

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Humans are exposed to a wide variety of chemicals from different sources, in both environmental and occupational settings. Numerous chemicals have been shown to exert endocrine disrupting properties, affecting normal function of the endocrine system. These are known as endocrine disrupting chemicals (EDCs). EDCs significantly impact public health by contributing to adverse health outcomes, including disrupted hormonal functions, impaired reproductive health, and metabolic diseases. Most of the epidemiological studies examining the adverse health effects of disrupted hormonal functions measure the levels of endogenous hormones and their carrier proteins and therefore may miss hormonal effects of EDCs. The endogenous hormones are traditionally measured by radioimmunoassay, enzyme-linked immunosorbent assay, and tandem mass spectrometry (MS/MS) and therefore lack any information on the biological effects of chemical exposure. As a result, novel methods that simultaneously capture both endogenous and exogenous chemicals modulating the hormone receptor signaling provide a better assessment of the combined effects of chemical exposure on hormonal functions. Herein, we show that the Chemical-Activated LUciferase gene eXpression (CALUX) assay is useful and can be used in epidemiologic research as an alternative method to measure total biological effects of chemical exposures on hormone receptor function. This dissertation aims to assess the effects of chemical exposure and other factors including race/ethnicity, alcohol consumption, and Indigenous American ancestry on hormone receptor bioactivities in human plasma/serum. Chapter 1 provides a review of the broadly relevant literature, including the endocrine system, functions of the hormone receptors, EDCs, factors modulating hormonal functions, and adverse health outcomes associated with EDC exposure. Chapter 2 focuses on a study that applied the estrogen receptor (ER)- and androgen receptor (AR)-mediated CALUX assays to measure serum estrogenic and androgenic bioactivities in workers occupationally exposed to trichloroethylene (TCE). This study not only provides evidence that occupational exposure to chemicals affects hormone receptor function, but it also is the first study to demonstrate that TCE is associated with elevated estrogenic bioactivity, which warrants further research on TCE and the estrogen related pathway. In Chapter 3, the CALUX assays were applied to assess the serum estrogenic, androgenic, and glucocorticogenic bioactivities in formaldehyde (FA) exposed workers. This study indicates that FA exposure does not affect hormone bioactivity measured in exposed workers. These TCE and FA human studies demonstrate the feasibility of CALUX assays on measuring hormone bioactivity in

occupationally exposed subjects. Chapter 4 applies the CALUX assay to explore the influence of demographic and lifestyle factors on plasma glucocorticogenic bioactivity in a larger study of 503 women who participated as controls in the San Francisco Bay Area Breast Cancer Study. This study reports that race/ethnicity, alcohol intake and Indigenous American ancestry influence plasma glucocorticogenic bioactivity, which could explain observed disparities in disease outcomes. Finally, in Chapter 5, the main findings of this dissertation are summarized and future research is suggested.

Dedicated to my family, without whom I am nowhere.

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Chapter 1

General introduction

1.1. Endocrine system

The endocrine system is a complex network including organs, tissues, and cells that are capable of producing hormones. Hormones are biomolecules synthesized and transported through systemic circulation to exert their functions at the target tissues. Proper hormone levels are fundamental for maintaining homoeostasis and normal physiology. Hormones can be generally categorized into 3 main groups based on their structures: amine hormones, peptide hormones, and steroid hormones. An amine hormone is derived from the modification of a single amino acid such as melatonin, dopamine, and thyroid hormone. Secondly, small-peptide-derived hormones are synthesized mainly on the rough endoplasmic reticulum inside cells. Their structures are usually more complex than the simple amine hormones. Luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) are all classic examples of peptide hormones. Lastly, a cholesterol derived hormone - as indicated by its name - is chemically formed from lipids and cholesterol in the body. Cholesterol derived hormones include estradiol, estrone, testosterone, dihydrotestosterone (DHT), and cortisol. Once hormones are synthesized, they are involved in various processes such as supporting reproductive development, metabolism, and growth. Multiple research studies have shown that exposure to endocrine disrupting chemicals (EDCs), which EDCs have structures similar to endogenous natural hormones, can disrupt hormone synthesis, metabolism, and transportation [1-3]. The main focus of this dissertation is on the effects of chemical exposure from both environmental and occupational sources on the hormonal functions of estrogen, androgen, and glucocorticoid steroid receptors.

1.1.1. Hormones of the Hypothalamic-Pituitary-Gonadal axis

1.1.1.1. The Hypothalamic-Pituitary-Gonadal axis

The Hypothalamic-Pituitary-Gonadal (HPG) axis is one of the endocrine pathways most sensitive to the effects of EDC exposure. Endocrine and reproductive functions are controlled by a hormone regulator called gonadotropin-releasing hormone (GnRH), which is secreted from the hypothalamus into the portal capillary. In the anterior pituitary gland, GnRH binds to its receptors and stimulates the synthesis and secretion of gonadotrophins (LH and FSH) into the systemic circulation [4]. These gonadotrophins control the development and maturation of follicles in the ovary (females) and sperm in the testis (males) [5, 6].

In the testis, Leydig cells are the major producers of androgens such as testosterone, DHT and androstenedione [7]. Elevated testosterone levels suppress the pituitary secretion of LH and FSH [8] in a negative feedback manner (Figure 1). Apart from androgens, estrogens can also be synthesized by spermatogenic cells and somatic cells in the testis [9]. The production of estrogens from androgens is governed by cytochrome P450 aromatase enzyme, which is responsible for producing estrogens through an irreversible conversion of androgens[10]. In addition to androgens, estrogens are also important in maintaining reproductive and hormonal functions in men [9, 10].

In the ovary, FSH stimulates the development and maturation of a dominant follicle, which plays a major role in estradiol production in females. The circulating estradiol concentrations are controlled through a feedback mechanism and are responsible for the surge in LH and FSH levels. LH stimulates ovulation and the formation of the corpus luteum, which in turn activates progesterone production and secretion [11] (Figure 1). The HPG axis, androgens, and estrogens are of critical importance for normal reproductive functions and sexual development. Moreover, the regulation of the endocrine and reproductive systems is a complex and highly coordinated process. Thus, a dysfunction in one axis has consequences at the other levels [3, 12].

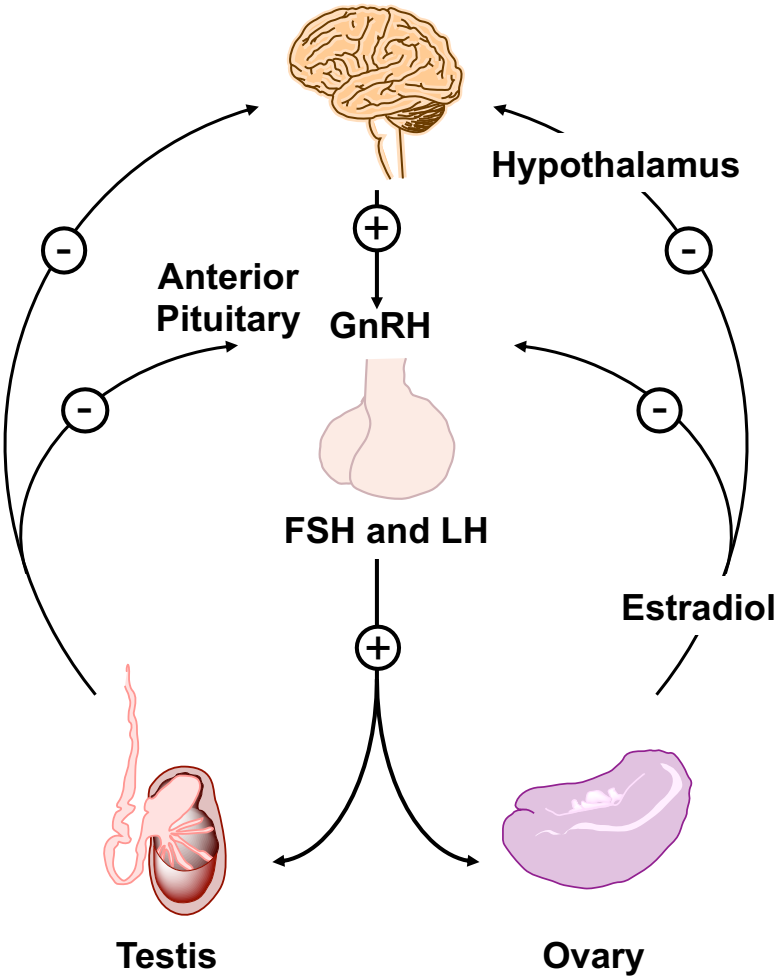

Figure 1. Hypothalamic-Pituitary-Gonadal axis.

1.1.1.2. Estrogen signaling and functions

The physiological effects of estrogens are exerted through the binding of estrogens to the estrogen receptor (ER). Binding of the receptor can be divided into two pathways: non-genomic signaling and genomic signaling. For the non-genomic signaling, estrogens bind to ERs localized on the plasma membrane, leading to an activation of fast signaling pathways in response to the stimuli. In the genomic estrogen signaling pathway, estrogens bind to both forms of ER, ERα and ERβ subtypes, localized in the cytosol and nucleus. The binding of estrogens to the ER facilitates receptor dimerization, trans-localization, and recognition of the estrogen response element (ERE) in the DNA sequence, therefore mediating the genomic effects. Co-activators and co-repressors are recruited to induce or suppress estrogenic gene expression [13]. In several tissues, ER controls growth, differentiation, and proliferation/apoptosis of normal cells [14]. Estrogens have been shown to regulate the expression of a large number of reproductive genes which control normal physiology of reproductive organs in both males and females [15, 16].

1.1.1.3. Androgen signaling and functions

Androgens play an essential role in the regulation of normal development and physiological processes, especially in the male reproductive system [17]. DHT is the most biological active androgen after its conversion from testosterone by an enzymatic reaction mediated by a P450 family member enzyme, 3-oxo-5α-steroid 4-dehydrogenase, commonly known as 5α-reductase. Several endogenous cholesterol metabolites have been shown to exhibit various degrees of androgenic activity [18]. The major biological effects of androgens are mediated through androgens binding to the androgen receptor (AR) [17]. AR is a member of the nuclear receptor gene superfamily and acts as a ligand-dependent transcription factor [19]. It functions as a regulator of downstream androgen dependent signaling cascades. The androgenic ligand-bound AR transcriptionally regulates the expression of androgen target genes. However, unlike estrogen signaling, AR in the absence of androgen ligands, is localized exclusively in the cytoplasm and forms a complex with heat-shock proteins, whereas ER is localized in both the cytoplasm and nucleus. Androgens binding to AR facilitates dissociation of AR from the heat-shock proteins and formation of androgenic ligand AR complex. In the nucleus, this complex forms a receptor dimerization and transcribes the androgen response element (ARE), which is responsible for many of the peripheral effects of androgens, such as promoting muscle growth, normal reproductive function, and secretion of seminal fluid [20, 21].

1.1.2. Hormones of the Hypothalamic-Pituitary-Adrenal axis

1.1.2.1. The Hypothalamic-Pituitary-Adrenal axis

The stress response system is highly controlled by the hypothalamic pituitary adrenal (HPA) axis [22]. The HPA axis is responsible for the neuroendocrine response to stressors or stress stimuli (Figure 2). In response to stimuli, the hypothalamus releases corticotropin-releasing hormone (CRH), which binds to CRH receptors on the anterior pituitary gland. As a result of the CRH binding, adrenocorticotropic hormone (ACTH) is released [22] and binds to its receptor located on the adrenal cortex and further stimulates adrenal release of cortisol into blood circulation [23]. The levels of cortisol are highest shortly after waking and decline rapidly in

subsequent hours after the awakening surge and then gradually drop throughout the day, reaching a nadir around bedtime [24]. The diurnal cortisol levels vary depending on several factors like stressor exposure. The circulating levels of cortisol are controlled by a negative feedback mechanism through the release of hypothalamic CRH and the pituitary release of ACTH. With chronic exposure to stress stimuli, our body habituates to the stimuli with sustained HPA axis activation, which can lead to adverse health outcomes.

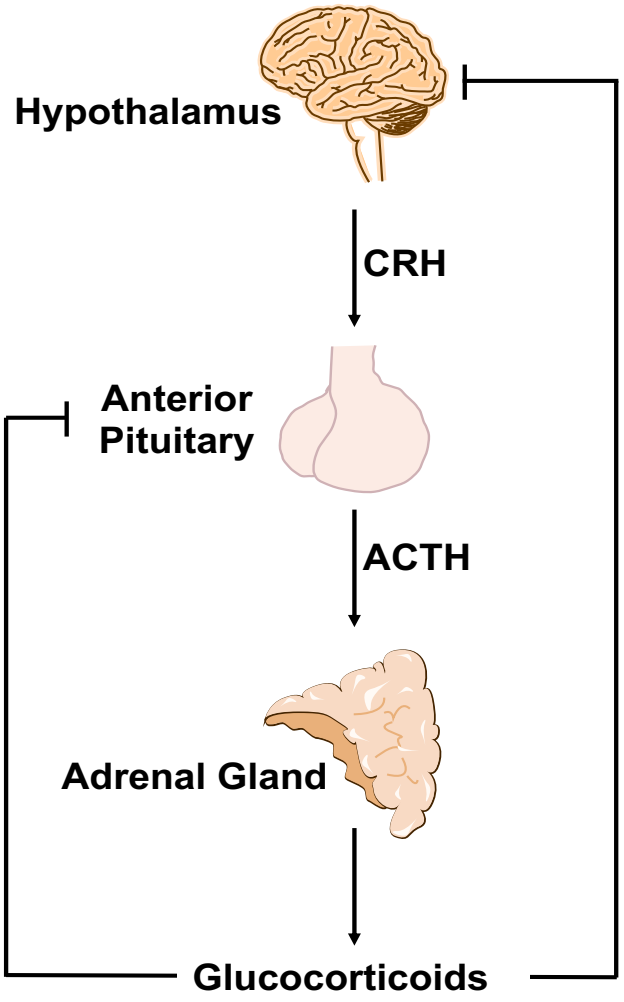

Figure 2. The regulatory response of glucocorticoid levels by the Hypothalamic-Pituitary-Adrenal axis.

1.1.2.2. Glucocorticoid signaling and functions

Glucocorticoids are primary stress hormones responsible for the regulation of numerous physiological responses in an effort to maintain homeostasis. The physiological actions of cortisol are exerted through the binding of the glucocorticoid receptor (GR), which belongs to the classical nuclear receptor superfamily of ligand-dependent transcription factors [25]. In the absence of glucocorticoids, GR localizes mainly in the cytoplasm as part of a large protein

complex with heat shock proteins (hsp90, hsp70, and hsp56), immunophilins, and p23 [26, 27]. These proteins maintain the GR in an inactive conformation. GR localizes in the nucleus upon glucocorticoid binding to the GR. GR then binds to glucocorticoid response elements (GREs) and transcribes the target genes [28], which regulate physiological functions including metabolism, immune function, skeletal growth, cardiovascular function, reproduction, and cognition [29, 30]. Recent studies have shown that prolonged exposure to glucocorticogenic compounds from both endogenous and exogenous origins has been associated with negative health outcomes such as breast cancer progression, psychotic symptoms, and depression [31-33].

1.2. Endocrine disrupting chemicals

The term EDC was first coined in the early 1990s. The World Health Organization (WHO) defines an EDC as "an exogenous substance or mixture that alters function of the endocrine system and consequently causes adverse health outcomes in an organism, or its progeny, or (sub) populations" [34]. Several EDCs have been shown to bind to nuclear receptors at the same binding pocket as natural endogenous ligands and stimulate or inhibit signal transduction responses. There are several receptors, especially ER [35], AR [36], GR [37, 38], progesterone receptor [39], mineralocorticoid receptor [38], thyroid receptor [40], peroxisome proliferator-activated receptor [41], which have been shown to be affected by EDCs. Furthermore, EDCs may interact with carrier proteins and impair the peripheral activity of the transported hormones [42]. Some EDCs alter biological activity or expression of metabolizing enzymes such as CYP19A aromatase, 5-reductase, and 17β-hydroxysteroid dehydrogenase [35, 43]. In addition, EDCs are capable of disrupting the synthesis and metabolism of natural hormones [44, 45]. The sources of EDC exposure include industrial chemicals, environmental contaminants, pharmaceuticals, naturally occurring compounds, pesticides, and plasticizers.

1.2.1. Exposure to endocrine disruptors

1.2.1.1. Occupational exposure to endocrine disruptors

Exposure to EDCs in the workplace is of high concern as the concentration and duration of occupational exposures to chemicals is generally much greater than those to the general public. However, little effort has been made to study the effect of occupational chemical exposures on hormonal functions.

1.2.1.1.1. Formaldehyde

Formaldehyde (FA) is a volatile chemical extensively used in industry and commerce as a cross-linking agent. Emerging evidence supports an association between FA exposure and several adverse health outcomes, particularly cancers. FA has been classified as a Group 1 human carcinogen, with sufficient animal experimental and epidemiologic evidence, by the International Agency for Research on Cancer (IARC) [46, 47] and has been evaluated by other independent agencies including the US National Toxicology Program (NTP) [48] and the US Environmental Protection Agency (U.S. EPA). Specifically, FA was classified as a human carcinogen based on epidemiologic evidence for the association with nasopharyngeal cancer. Although the adverse health effects regarding the cancer-related outcomes are well studied, little

is known about the endocrine disrupting properties of FA in humans.

Only a few studies have been conducted to examine the effect of FA exposure on the endocrine system [49]. However, findings in animal studies related to the production of hormones are inconsistent. Some studies found no notable association between FA exposure and abnormal reproductive hormone levels. Zhou reported that male Sprague-Dawley male rats exposed to FA at 2.46 mg/m^3 through inhalation had abnormal sperm parameters and decreased testicular seminiferous tubular diameter; however, serum testosterone levels were not altered [50]. In addition, in female rats, serum estrogen levels were not affected by FA exposure [51]. Khalil et al., found that male Swiss mice administered with FA at 25 mg/kg/day had slightly lower testosterone and LH due to the reduction in testicular steroidogenic gene expression such as StAR, P450scc, and 3β-HSD-1 genes [52].

Only one human study has investigated the effects of FA exposure on the production of reproductive hormones. Chinedu et al., reported that short-term exposure to FA was associated with significant alterations in serum testosterone and FSH levels, but no significant changes in LH, estradiol, and prolactin were observed [49].

1.2.1.1.2. Trichloroethylene

Trichloroethylene (TCE) is a volatile solvent used in a large spectrum of applications in industry to remove grease from metals [53-55]. TCE significantly contaminates air from the source of emission [56] and can be found in soil, surface and groundwater supplies via industrial water discharge and leaching from landfills or underground storage tanks [57-59]. Countries with the greatest consumption of TCE are the United States, India, and China [60, 61]. The widespread use and high demand of TCE has contributed to environmental contamination and concerns of adverse health hazards in both the occupational setting and in the population at large [62-64].

There are very limited in vitro studies highlighting the potential endocrine disrupting effects of TCE and its metabolites, trichloroacetic acid (TCA) and trichloroethanol (TCOH). Only a few studies are available to suggest that TCE and its metabolites function as EDCs through disruption of reproductive hormones in cell culture [43, 65, 66]. In a primary cell culture of Leydig cells, TCE and TCA stimulated the production of estradiol in a dose-dependent manner [65]. However, a prominent reduction in testosterone levels was observed in the TCE and TCA exposed groups. Furthermore, studies showed that, at biological concentrations that can be achieved in exposed workers, TCE and its major metabolites exhibited endocrine disrupting effects in vitro [43, 60, 66]. Increased production of estradiol was significantly increased in the exposed groups, and was attributed to upregulation of 17β-hydroxysteroid dehydrogenase, an enzyme involved in the production of estradiol [43]. These mechanistic studies support the notion that TCE can be an endocrine disruptor.

Only a few animal studies have investigated the effects of TCE on reproductive outcomes. Kumar et al., [67, 68] reported effects of TCE on sperm count and sperm motility in rats administered by the inhalation route. Male Wistar rats were exposed to 376 ppm of TCE for 12 and 24 weeks. Reductions in epididymal sperm count were observed at both time points in exposed rats versus controls [67, 68]. In addition, a decrease in serum testosterone and testicular

17β-hydroxy steroid dehydrogenase was observed in TCE exposed rats. Importantly, there was increased severity of the effects as exposure duration increased [67, 68].

A number of epidemiological studies have been conducted to investigate the effects of TCE on endocrine and reproductive parameters following occupational and community exposures. Epidemiological studies of male reproduction examined altered sperm morphology, infertility, and hormone levels related to TCE exposure. However, the results reported from different studies were inconsistent. A cross-sectional occupational study compared sperm morphology of 85 men exposed to TCE at high (urine TCA ≥ 25 mg/g creatinine) and low (urine TCA < 25 mg/g creatinine) concentrations. The urine TCA levels were adjusted with urine creatinine and used as a biomarker of TCE exposure. A decreased percentage of normal sperm morphology was observed in the high-exposure group compared with the low-exposure group [69]. On the other hand, a number of epidemiological studies reported no association between TCE exposure and adverse male reproductive outcomes including sperm count and sperm morphology [70, 71].

1.2.1.2. Environmental exposure to endocrine disruptors

Environmental exposure to EDCs that can perturb the endocrine system may do so by acting directly upon any level of the HPG axis and hormone receptors or via indirect actions upon transportation, metabolism and synthesis of the hormones [3, 12, 72]. Several epidemiological studies examining the effects of EDC exposure showed that EDCs perturb at least one key hormone of the HPG axis [3, 73-75]. For instance, a study investigating serum phthalates in general populations from Greenland, Poland, and Ukraine revealed that serum phthalates were negatively associated with sperm count, testosterone, and SHBG [12]. Other studies have shown that several environmental contaminants exert endocrine disrupting capabilities and cause adverse effects to both humans and wildlife. Zhang et al. recently reported that fluorene-9-bisphenol (bisphenol A substitute) exhibited a strong anti-estrogenic effect through the ER-mediated pathway, causing developmental and reproductive toxicity in mice with sub-chronic exposure. Mice exposed to fluorene-9-bisphenol at human exposure levels caused disruption in the estrogen sensitive gene expression in uteri. Altogether, the evidence suggests that exposures to some environmental contaminants can disrupt the endocrine system and cause adverse health outcomes.

1.3. Factors affecting endocrine system and endogenous hormone levels

1.3.1. Alcohol consumption

Numerous studies have reported that alcohol consumption affects the HPA axis and the stress-response mechanism [76, 77]. Specifically, high alcohol consumption stimulates the HPA axis, which leads to an increase in serum levels of ACTH and glucocorticoids [76, 77]. Plasma cortisol levels were significantly increased in healthy subjects administrated a high dose of alcohol [78]. Similarly, Thayer et al., reported that healthy men in the highest tertile of self-reported alcohol intake had greater urinary cortisol levels compared to men in the lower tertiles [79]. Moreover, alcoholics had about three times greater hair cortisol levels than abstinent alcoholics or non-alcoholics [80]. These findings substantiate previous studies on dysregulation of the HPA axis and suggest impaired negative-feedback of the HPA axis in heavy drinkers. Similar

findings were observed in several in vivo studies, where acute alcohol exposure in rats increased plasma levels of ACTH and corticosterone mediated by the enhancement of CRF release from the hypothalamus [81]. Moreover, alcohol consumption also disrupts the production of reproductive hormones and the HPG axis [82]. Chronic alcohol consumption has been found to be associated with a wide range of health effects, including reduced libido, testicular atrophy and increased risk of infertility, disrupted menstrual cycles, delay of ovulation, and spontaneous abortions.

1.3.2. Smoking

Tobacco and cigarette smoke contains a wide variety of toxic chemicals including, nicotine, benzo-[a] pyrene, dimethynitrosamine, diethylnitrosamine, hydrazine, benzene, FA and arsenic [83]. These chemicals have been shown to alter physiological processes in the body including hormone synthesis and metabolism. Smoking affects the normal hormonal function of several endocrine organs such as the thyroid gland, adrenal gland, testis and ovary. Apart from nicotine, other toxic chemicals in cigarette smoke have been shown to exhibit effects on hormonal secretion and levels of endogenous steroid hormones. Kirchbaum et al., reported that acute and chronic smoking increased serum levels of cortisol [84]. Moreover, cortisol levels were found to be correlated with the number of cigarettes smoked per day [84]. Smoking can also alter concentrations of SHBG [85], free testosterone, and metabolism of circulating testosterone. Mittler et al., conducted in vivo experiments and found that animals chronically exposed to cigarette smoke had lower testosterone levels and lower hepatic enzymatic activity of 7-alpha-hydroxylases and 6-beta-hydroxylases (enzymes responsible to testosterone metabolism) suggesting that chronic exposure to smoking increased the hepatic metabolism of testosterone [86].

1.3.3. Obesity

Reductions in testosterone levels in plasma or serum have been reported in multiple studies of obese men [87, 88]. Obese men with a Body Mass Index (BMI) of 30 to 40 kg/m^2 possessed a 50% decrease in total testosterone concentrations compared to age-matched healthy controls, but no change in free testosterone levels was observed. In addition, serum concentrations of FSH and LH were not significantly different between the obese and control groups [87, 89]. A decrease in total testosterone levels was interpreted as reflecting obesity-associated reduction in SHBG, a carrier protein of testosterone [87, 89]. Nevertheless, other studies of men with a BMI higher than 40 kg/m^2 reported reductions in both total and free circulating testosterone levels along with decreased concentrations of LH [88].

Multiple studies have shown that circulating estrogen levels are associated with BMI and that estrogen production in adipose tissue may be increased by 10-fold in morbidly obese postmenopausal women [90, 91]. Interestingly, weight loss interventions in postmenopausal women have been associated with lower circulating estrogen levels, indicating a reversal of peripheral production of estrogens via reduction of adipose tissue.

1.3.4. Race and ethnicity

Apart from lifestyle factors like alcohol consumption and smoking, race and ethnicity

have been shown to influence levels of circulating hormones. Orwoll et al., analyzed concentrations of circulating serum androgens, estrogens, and sex steroid precursors/metabolites in 5,003 men from five different countries [92]. When categorized into different ethnic groups (Caucasian, Black, or Asian), Black men had higher DHT levels followed by Asian and White. Moreover, Black men had higher levels of estrogens (estradiol, estrone), and Asian men had lower levels of glucuronidated androgen metabolites after adjusting for age and BMI [92]. Other studies reported similar findings that Black men have higher testosterone levels compared to White men [93, 94]. Racial/ethnic differences in hormone levels are also observed in women. Non-Hispanic White (NHW) women had higher total and free estradiol and testosterone levels than Hispanics after adjusting for age, type of menopause, waist circumference, alcohol consumption, and current smoking status. NHW also had higher levels of free estradiol and lower levels of SHBG than Black women [95].

1.4. Research Objectives

My research objectives were to examine the effects of occupational exposure, demographic, and lifestyle factors including race/ethnicity, alcohol consumption, and indigenous American ancestry on hormone bioactivities in human biospecimens. My specific aims define the next four chapters of my dissertation as follows:

Chapter 2 – Examine the effects of TCE exposure on serum total estrogenic and androgenic activities using ER- and AR-mediated Chemical-Activated LUciferase gene eXpression (CALUX) assays.

Chapter 3 – Examine the effects of FA exposure on serum total estrogenic, androgenic, and glucocorticogenic activities using ER-, AR-, and, GR-mediated CALUX assays, respectively.

Chapter 4 – Investigate if demographic and lifestyle factors influence plasma glucocorticogenic activity using GR-mediated CALUX assays.

1.5. References

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Chapter 2

Assessment of the serum estrogenic and androgenic bioactivity in male workers occupationally exposed to trichloroethylene

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Abstract

Trichloroethylene (TCE) is a volatile chemical that is widely used in the manufacturing sector and is ubiquitously present in the environment. Several adverse health outcomes resulting from TCE exposure in humans have been described but little is known regarding the ability of TCE to disrupt the endocrine system. Previous epidemiological studies examining the effects of TCE exposure on hormonal function have serious limitations, including imprecise exposure assessment and potential confounding of exposure. To address these limitations, we conducted a cross-sectional molecular epidemiological study of male workers exposed to TCE (mediam of exposure is 13.9 ppm). The Chemical-Activated LUciferase gene eXpression (CALUX) assays were used to examine if TCE exposure affect total serum estrogenic (E) and androgenic (A) activities, measures that reflect levels of total serum estrogens and androgens, respectively. Results showed a 1.4-fold increase in the median serum E bioactivity in the TCE exposed group relative to controls (P=0.036). There was no significant difference in serum A bioactivity between the exposed group and controls. Our findings suggest that TCE is a potential human endocrine disruptor and is associated with altered serum estrogen bioavailability. Future research should focus on the endocrine disrupting effects of TCE exposure, particularly those involving estrogen-related pathways.

2.1. Introduction

Trichloroethylene (TCE) is a volatile compound extensively used in industrial applications to clean oil and grease from manufactured metals and electronic circuits [1]. The widespread use of TCE has contributed to environmental contamination [2, 3] leading to elevated heath concerns in both occupational settings and in the population at large.

Previous studies have shown that TCE exposure are associated with several adverse health outcomes, including hematotoxicity [4] and cancers [5, 6]. Furthermore, we previously reported that workers occupationally exposed to TCE had lower lymphocyte, T cell, CD8+ T cell, and natural killer cell counts [4]. Although there is sufficient knowledge regarding the haematotoxicity and cancer-related outcomes, a limited number of studies have examined whether TCE exposure is associated with disrupted endocrine function in humans.

In mechanistic studies using in vitro approaches, TCE and its metabolites have been shown to elicit notable endocrine disrupting effects. In a primary cell culture of Leydig cells, TCE and trichloroacetic acid (TCA), a major metabolite of TCE, stimulated the production of 17β-estradiol in a concentration dependent manner [7]. Furthermore, studies reported that at biological concentrations, which can be achieved in occupationally exposed workers, TCE and its major metabolites exhibited endocrine disrupting effects in vitro [8-10]. Specifically, the production of 17β-estradiol was elevated in exposed groups attributed to an upregulation of 17β-hydroxysteroid dehydrogenase, an enzyme involved in the production of 17β-estradiol [8]. These mechanistic studies support the notion that TCE could function as an endocrine disruptor. However, the endocrine disrupting effects of TCE, in particular on estrogen-related pathways have yet to be elucidated in epidemiologic research.

Chemical-Activated LUciferase gene eXpression (CALUX) assays have been widely used in multiple epidemiologic research studies to determine the effects of environmental chemical exposures on hormone receptor function [11-13]. CALUX assays detect total biological activity of all endogenous and exogenous compounds acting on hormone receptors. Thereby, these assays provide insight into a response that captures internal and external exposures from both biological and environmental inputs.

Previous epidemiological studies examining the effects of TCE on hormonal functions have serious limitations regarding exposure assessments and potential confounding exposures. Generally, studies do not assess the effects of the individual chemical, which makes it difficult to assess the impact of TCE on health outcomes. To address these limitations, we conducted two cross-sectional molecular epidemiological studies of male workers exposed to TCE (n=86) in Guangdong, China. We investigated the total serum E and androgenic (A) bioactivities, measures of total serum androgens, including testosterone and dihydrotestosterone, and total serum estrogens, including 17β-estradiol and estrone, in the exposed workers. Additionally, we examined the correlations between serum E and A activities and previously reported haemato-immunological parameters in the same study population [4].

2.2. Materials and Methods

2.2.1. Study population and exposure assessment

The study designs and exposure assessments of the cross-sectional molecular epidemiological study have been described in detail elsewhere [4]. In the TCE study, 57 exposed male workers were recruited from six workplaces that currently used TCE in the manufacturing process and the control group, comprised of 29 male workers, were recruited from different workplaces within the same geographic area in Guangdong, China and did not use TCE in their manufacturing process. Personal air exposure assessments using a 3M organic vapor monitor 3500 were performed. The TCE exposure assessment was collected from exposed workers over the course of a full work shift during a 3-week period prior to blood sample collection. Monitoring of TCE levels was performed in these workplaces during an initial screening and there were no other chemical exposures that might be potential confounders, for example benzene, phenol, styrene, ethylene oxide, pichlorohydrin, and chlorinated solvents.

All study participants were administered a questionnaire that assessed information on lifestyle and demographic characteristics, environmental exposures, occupational and medical history, and current tobacco as well as alcohol use. Workers with a history of cancer or a previous occupation with notable exposure to benzene, butadiene, and styrene were not included in the study. Lastly, blood samples were collected from all workers.

2.2.2. Serum estrogenic bioactivity measurements

CALUX assays were used to simultaneously assess the total serum E and A bioactivity profiles. The bioactivity profiles provide a summated measure of biological effects encompassing both endogenous and exogenous compounds activating the hormone receptors. Methods followed procedures as described in previous studies [14]. Lab personnel who performed the CALUX assays were blinded to the TCE exposure status of the samples.

ER-mediated CALUX assay was used to measure serum E bioactivity. T47D-Kbluc cell line (ATCC®CRL-2713™), a human breast cancer cell line stably transfected with a luciferase reporter gene construct under transcriptional regulation of triplet estrogen response elements, was obtained from the American Type Culture Collection (ATCC). Cells were cultured in phenol red DMEM (Gibco, Grand Island, NY) prior to performing the luciferase assay as previously described[14]. Cells were then seeded at a density of 3.0×10^4 cells/well in white, 96-well microtiter plates (Thermo Scientific, Grand Island, NY, USA) and incubated for 24 hours. After the incubation period, 20 μL of serum per sample was diluted in phenol red free medium containing 10% charcoal-dextran stripped fetal bovine serum (FBS, Corning, Bedford, MA) and added in quadruplicate directly onto cells. After 24 hours of incubation, cells were lysed with 1× passive lysis buffer (Promega, Madison, WI, USA) and the microplate was read using a luminometer (Berthold Technologies, Oak Ridge, TN, USA). Readings from each well were reported in relative light units (RLUs) with higher RLU values indicating greater serum E bioactivity. The RLUs from quadruplicate wells were averaged to get one measurement of E bioactivity per individual. The overall intra-assay and inter-assay coefficients of variation (CVs) of this assay were 5–19 %. The minimum detection limit for 17β-estradiol was 1.5×10^{-12} M.

2.2.3. Serum androgenic bioactivity measurements

Similarly, an androgen receptor (AR) mediated CALUX assay was used to measure serum A bioactivity. The MDA-kb2 cell line (ATCC®CRL-2713™), a human triple negative breast cancer cell line stably transfected with the murine mammalian tumor virus (MMTV) luciferase neo reporter gene construct, was obtained from the ATCC. Because AR and glucocorticoid receptor have homologous DNA binding domains and act on the same MMTV promoter, this cell line has the ability to measure both androgens and glucocorticoids in biospecimens. To distinguish between A and glucocorticoid activities, we used a potent AR inhibitor, hydroxyflutamide (OHF, Sigma-Aldrich, St. Louis, USA). MDA-kb2 cells were routinely maintained in Leibovitz's-15 (L-15) medium (Gibco, Grand Island, NY, USA) as describe previously [14]. The cells were then seeded at a density of 3.0×10^4 cells/well in white, 96-well microtiter plates (Thermo Scientific, Grand Island, NY, USA) and incubated at 37°C for 24 hours. After this initial incubation period, the 15 µL serum per sample was diluted in L-15 medium containing 10% charcoal-dextran stripped FBS and then added in quadruplicate directly onto cells, both in the presence and absence of 5×10^{-7} M OHF. The A bioactivity measurements were obtained by subtracting the averaged RLUs from quadruplicate wells in the absence of OHF from the averaged RLUs in the presence of OHF per sample. The intra-assay and inter-assay CVs of this assay are 4–12%. The minimum detection limit for testosterone mimic is 1.25×10^{-10} M.

2.2.4. Ethical statement

The study was approved by the Institutional Review Boards at the U.S. National Cancer Institute and the Guangdong Poison Control Center in China and all study participants provided written informed consent.

2.2.5. Statistical analysis

All statistical analyses were performed using Statistical Analysis Software (SAS v. 9.1.3, Cary, NC). Previously conducted analyses have been described elsewhere[4]. Briefly, unadjusted means and standard deviations were calculated for all endpoints. Linear regression using the natural logarithm of each endpoint was used to test for differences between workers exposed to TCE and control workers. All regression models were adjusted for age (continuous variable). Further adjustment for current cigarette smoking (yes/no), current alcohol consumption (yes/no), recent respiratory infection (yes/no) and body mass index (BMI) was conducted if the regression coefficient of the corresponding exposure variable, was altered by more than 15% change. The correlation between serum hormone bioactivities and previously reported haemato-immunological parameters was carried out using Spearman's correlation analysis.

2.3. Results

In the TCE study, study participants were divided into two groups: controls and TCE exposed group (median of TCE levels = 13.9 ppm). Demographic characteristics including age, alcohol consumption, smoking status, recent infection status and BMI were comparable among the exposed and control groups (Table 1).

Exposure to TCE resulted in a 40 % increase in the average of serum E bioactivity (a 75 % increase in the median of serum E bioactivity) relative to controls (P=0.036 for exposed vs. control groups), adjusted for age (Figure 1). In contrast, serum A bioactivity in the exposed group was not different than the controls (P=0.831), after adjusting for age and alcohol consumption (Figure 2). Moreover, serum E bioactivity was significantly and positively correlated with complete blood counts of leukocytes, granulocytes, and monocytes. Furthermore, serum A bioactivity was significantly and positively correlated with serum immunoglobulin M (IgM) levels in the TCE study. No correlations between serum E and A activities and other hemato-immunological parameters were observed (Table 2).

2.4. Discussion

Previous epidemiological studies examining the effects of TCE on endocrine function relied primarily on occupational and community exposure to a mixture of organic solvents containing TCE, which can misconstrue health effects associated solely with TCE exposure. To the best of our knowledge, there has been no occupational epidemiological study to date that addressed endocrine disrupting effects of TCE, without co-exposures to other chemicals exposures. We minimized such potential confounders and bias by using personal monitoring data that provided TCE exposure measurements and documented that other airborne exposures were negligible.

We found that TCE exposure did not affect serum A bioactivity, consistent with a previous epidemiologic study of 85 workers, where levels of TCE exposure were not associated with the levels of serum testosterone [15]. However, in animal studies, male Wistar rats exposed to 376 ppm of TCE for 12 and 24 weeks had lower serum testosterone and testicular 17β-hydroxy steroid dehydrogenase [16]. The severity of the adverse effects increased with increasing exposure duration to TCE [16]. Discrepancies between studies may reflect differences in species as well as the magnitude of TCE exposure, which was 30 times greater in the Wistar rats (376 ppm) than in the exposed workers (13.9 ppm).

Although previous epidemiological studies of chemical exposure in men primarily focused on alterations to male reproductive hormones like testosterone, dihydrotestosterone and androstenedione, we emphasize the significance of estrogens as a potential target of endocrine disruption. Our results showed that workers exposed to TCE had elevated serum E bioactivity, a finding that is consistent with previous mechanistic in vitro studies [7, 8]. Exposure to TCE and TCE metabolites caused a significant increase in 17β-estradiol production in vitro as a result of an upregulation in of 17β-hydroxysteroid dehydrogenase, an enzyme involved in the production of 17β-estradiol [8].

Even though TCE has been reported to promote male infertility in humans, there is limited mechanistic evidence supporting this observation [17, 18]. The central role of 17β-estradiol in the regulation of male infertility has been verified in both animals and humans, [19, 20] which showed that an elevation of serum 17β-estradiol promoted engulfment of Leydig cells by macrophages, leading to male infertility [19]. Clinical studies have revealed that treatment of infertile men with high serum estradiol/testosterone using an aromatase inhibitor, which inhibits the conversion of testosterone to 17β-estradiol, has proven successful in reversing infertility.

After drug administration, the patients showed significant improvement in semen parameters and levels of testosterone and 17β-estradiol returned to normal [20-22]. Here we provide additional evidence that TCE exposure altered the balance between 17β-estradiol and testosterone and significantly increased serum E bioactivity.

TCE has been associated with a variety of immunotoxic effects and may be associated with increased risks of some autoimmune and hypersensitivity disorders as well as non-Hodgkin lymphoma (NHL) [23, 24]. Not only do reproductive tissues express estrogen receptors (ERs), several human hematopoietic cells such as lymphocytes, natural killer cells, neutrophils, and bone marrow-derived macrophages also express ERs [25-27]. Previous studies have reported that serum estrogens regulate hematopoietic and immune cells [27-29]. Moreover, the associations between obesity and NHL as well as multiple myeloma have been mediated through estrogens. In the present study, we found that serum E bioactivity was significantly correlated with the absolute cell counts of granulocytes, leukocytes, and monocytes. These results substantiate previous reports of correlations between serum estrogen and altered blood immune cells [28]. Our findings suggest that TCE exposure may affect immunotoxicity through an ER mediated mechanism.

Our study had some limitations. Aside from the small sample sizes, we did not account for measures of endogenous estrogens and androgens, which could have differed between TCE-exposed workers and controls. However, this limitation is partially offset by our measurements of total serum E and A activities, which reflect effects of both exogenous and endogenous molecules.

2.5. Conclusion

This is the first study to report that TCE exposure may alter human male serum estrogen bioavailability. Moreover, we found that serum E bioactivity correlated with some haemato-immunological parameters. Future studies of effects of TCE exposure should consider the estrogen related pathway as a potential target for endocrine disruption.

2.6. References

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Figures and tables

Table 1. Demographic characteristics of controls and workers exposed to trichloroethylene in a cross-sectional molecular epidemiology study conducted in Guangdong, China.

Characteristics	TCE	
	Control	Exposed
	n = 29	n = 57
Age, mean (SD) [a]	24.8(5.8)	25.7(6.9)
BMI, mean (SD) [a]	21.3(2.9)	21.6(2.7)
Current alcohol use, n (%)		
Yes	15(51.7)	23(40.4)
No	14(48.3)	34(59.6)
Current smoke [b], n (%)		
Yes	15(51.7)	34(59.6)
No	14(48.3)	23(40.4)
Recent infection [b], n (%)		
Yes	2(6.9)	9(15.8)
No	27(93.1)	48(84.2)
Median exposure, ppm	0.025	13.9

BMI, body mass index.
[a]Mean ± standard deviation.
[b]Number (percent).

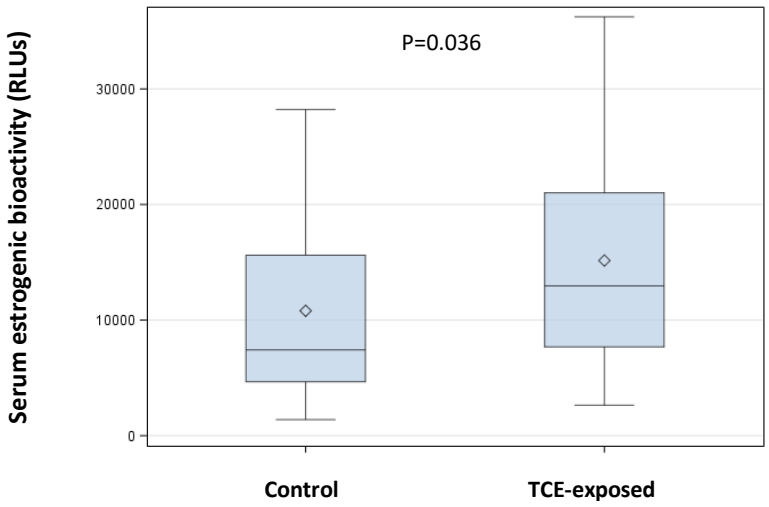

Figure 1. Box and whisker plots, depicting the median (line), lower and upper interquartile range (IQR; box), mean (open squares) and whiskers to the highest and lowest values of average serum estrogenic bioactivities by trichloroethylene (TCE) exposure category. P-values between controls and the exposed group for serum estrogenic bioactivity is 0.036. Levels of serum estrogenic bioactivity was adjusted for age.

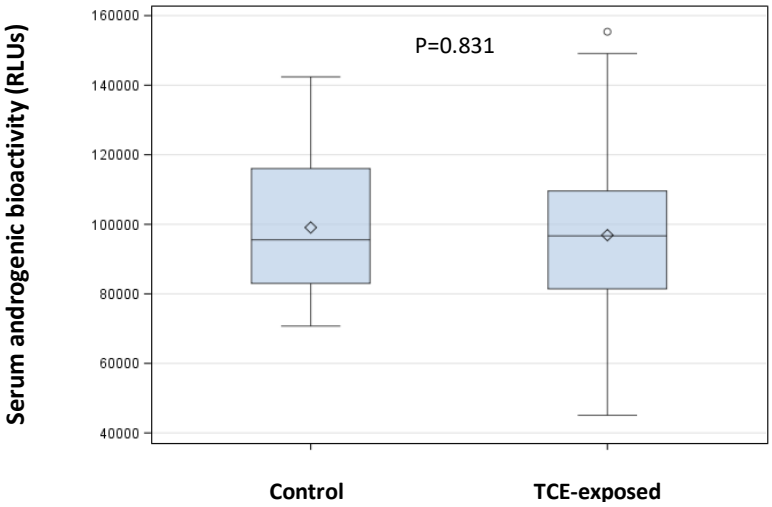

Figure 2. Box and whisker plots, depicting the median (line), lower and upper interquartile range (IQR; box), mean (open squares) and whiskers to the highest and lowest values of average serum androgenic bioactivities by trichloroethylene (TCE) exposure category. P-values between controls and the exposed group for serum androgenic bioactivity is 0.831. Levels of serum androgenic bioactivity was adjusted for age and alcohol consumption.

Table 2. Correlations coefficient of serum estrogenic and androgenic bioactivities with haemato-immunological parameters in male workers exposed to trichloroethylene.

Hemato-immunological parameters	Serum estrogenic activity	Serum androgenic activity
Leukocyte	0.40****	0.05
Granulocyte	0.38***	0.03
Monocyte	0.27*	-0.04
Lymphocyte	0.17	0.13
IgG	-0.06	0.06
IgM	-0.11	0.23*

Numbers of subjects = 86; *P <0.05, ***P < 0.001, ****P < 0.0001.

Chapter 3

No Association between Occupational Exposure to Formaldehyde and Disrupted Serum Hormone Receptor Activity

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Abstract

Formaldehyde (FA) is a volatile organic chemical widely used as a cross-linking agent in the manufacturing sector. FA has been reported to cause a number of toxic effects, but little is known regarding the ability of FA to disrupt the endocrine system in humans. Data from previous studies that assessed the effects of FA exposure on hormone levels are inconsistent. We conducted a cross-sectional molecular epidemiological study of male workers occupationally exposed to FA. We used Chemical-Activated LUciferase gene eXpression (CALUX) assays to examine the effects of FA exposure on total serum estrogenic (E), androgenic (A), and glucocorticogenic (G) bioactivities, which reflect levels of total serum estrogens, androgens and glucocorticoids, respectively. Moreover, we further explored the possible mechanism of endocrine disruption using TaqMan gene expression assay against all of the key steroidogenic genes in human adrenocortical carcinoma cell line. Results revealed that there was no significant difference in serum E, A, and G bioactivities between the exposed group and controls. Consistently, the results from the gene expression assay supported the findings in the FA human study as there was no change in the expression of any steroidogenic genes in the FA exposed groups. Our findings indicate that FA does not affect hormone receptor bioactivity in humans.

3.1. Introduction

Formaldehyde (FA) is a volatile compound extensively used in several manufacturing industries and is ubiquitously found in various environmental media[1, 2]. FA is utilized as a biological preserving medium and a precursor for the production of more complex compounds and materials, such as melamine resin, polyoxymethylene plastics, and butanediol[3]. The widespread use of these chemicals has contributed to environmental contamination leading to elevated heath concerns in both occupational settings and in the population at large.

Previous studies have shown that FA exposure is associated with several adverse health outcomes, including haematotoxicity[4, 5] and cancers[6]. FA has been classified as group 1 human carcinogen by the International Agency for Research on Cancer (IARC)[6, 7], with sufficient animal experimental and epidemiologic evidence and have been evaluated by other independent agencies including the US National Toxicology Program (NTP)[8] and the US Environmental Protection Agency (U.S. EPA). Although there is sufficient evidence regarding the cancer-related outcomes, little is known regarding the endocrine disrupting potential of FA in humans.

Human and animal studies investigating the effects of FA exposure on the production of reproductive hormones are limited. Among the few studies that are available, inconsistency exists among the findings related to the production of hormones. For examples, while some studies reported no association between FA exposure and altered reproductive hormone levels[9, 10], others found that serum testosterone levels were slightly decreased in FA exposed animals[11]. In addition, male Swiss mice administered with FA at 25 mg/kg/day had slightly lower testosterone and luteinizing hormone (LH) due to reductions in testicular steroidogenic gene expression[11]. In human studies, a short-term exposure to FA has been shown to be associated with significant alterations in serum testosterone and follicle stimulating hormone (FSH) levels, but no significant changes in LH, 17β-estradiol, and prolactin were observed[12].

The NCI-H295R cell line is a human adrenocortical carcinoma cell that expresses all major steroidogenic enzymes and has therefore, been used as the gold standard for assessing the direct effects of endocrine disruption on steroidogenesis. The use of the cell line renders the ability to measure alterations in gene transcription and hormone production [13, 14]

Previous human studies examining the effects of FA on hormonal functions have serious limitations regarding exposure assessments and potential confounding exposures. We conducted a cross-sectional molecular epidemiological study of male workers exposed to FA (n=81) in Guangdong, China. We investigated the total serum E, androgenic (A), and glucocorticogenic (G) bioactivities in the exposed workers.

3.2. Materials and Methods

3.2.1. Study population and exposure assessment

The study designs and exposure assessments of the cross-sectional molecular epidemiological study of FA have been described in detail elsewhere[4, 15]. In the FA study, 37 exposed male workers were recruited from two workplaces in Guangdong, China that used or

manufactured melamine and the control group consisted of 44 male workers who had no exposure to FA. Personal air exposure measurements using a SKC UMEx 100 passive samplers were performed. The exposure assessments were collected from exposed workers over the course of a full work shift during a 3-week period prior to blood sample collection. Monitoring of FA levels was performed in these workplaces during an initial screening and there were no other chemical exposures that might be potential confounders, for example benzene, phenol, styrene, ethylene oxide, pichlorohydrin, and chlorinated solvents.

All study participants were administered a questionnaire that assessed information on lifestyle and demographic characteristics, environmental exposures, occupational and medical history, and current tobacco as well as alcohol use. Workers with a history of cancer or a previous occupation with notable exposure to benzene, butadiene, and styrene were not included in the study. Lastly, blood samples were collected from all workers.

3.2.2. Serum estrogenic bioactivity measurements

Chemical-Activated LUciferase gene eXpression (CALUX) assays have been widely used in multiple epidemiologic research studies to determine the effects of environmental chemical exposures on hormone receptor function[16-18]. CALUX assays detect total biological activity of all endogenous and exogenous compounds acting on hormone receptors. Thereby, these assays provide insight into a response that captures internal and external exposures from both biological and environmental inputs. Methods followed procedures as described in previous studies[19]. Lab personnel who performed the CALUX assays were blinded to FA exposure status of the samples.

ER-mediated CALUX assay was used to measure serum E bioactivity. T47D-Kbluc cell line (ATCC®CRL-2713™), a human breast cancer cell line stably transfected with a luciferase reporter gene construct under transcriptional regulation of triplet estrogen response elements, was obtained from the American Type Culture Collection (ATCC). Cells were cultured in phenol red DMEM (Gibco, Grand Island, NY) prior to performing the luciferase assay as previously described[19]. Cells were then seeded at a density of 3.0×10^4 cells/well in white, 96-well microtiter plates (Thermo Scientific, Grand Island, NY, USA) and incubated for 24 hours. After the incubation period, 20 μL of serum per sample was diluted in phenol red free medium containing 10% charcoal-dextran stripped fetal bovine serum (FBS, Corning, Bedford, MA) and added in quadruplicate directly onto cells. After 24 hours of incubation, cells were lysed with 1× passive lysis buffer (Promega, Madison, WI, USA) and the microplate was read using a luminometer (Berthold Technologies, Oak Ridge, TN, USA). Readings from each well were reported in relative light units (RLUs) with higher RLU values indicating greater serum E bioactivity. The RLUs from quadruplicate wells were averaged to get one measurement of E bioactivity per individual. The overall intra-assay and inter-assay coefficients of variation (CVs) of this assay were 5–19 %. The minimum detection limit for 17β-estradiol was 1.5×10^{-12} M.

3.2.3. Serum androgenic and glucocorticogenic bioactivity measurements

Similarly, an androgen receptor (AR) mediated CALUX assay was used to measure serum A bioactivity. The MDA-kb2 cell line (ATCC®CRL-2713™), a human triple negative

breast cancer cell line stably transfected with the murine mammalian tumor virus (MMTV) luciferase neo reporter gene construct, was obtained from the ATCC. Because AR and glucocorticoid receptor have homologous DNA binding domains and act on the same MMTV promoter, this cell line has the ability to measure both androgens and glucocorticoids in biospecimens. To distinguish between A and glucocorticoid activities, we used a potent AR inhibitor, hydroxyflutamide (OHF, Sigma-Aldrich, St. Louis, USA). MDA-kb2 cells were routinely maintained in Leibovitz's-15 (L-15) medium (Gibco, Grand Island, NY, USA) as describe previously[19]. The cells were then seeded at a density of 3.0×10^4 cells/well in white, 96-well microtiter plates (Thermo Scientific, Grand Island, NY, USA) and incubated at 37°C for 24 hours. After this initial incubation period, the 15 μL serum per sample was diluted in L-15 medium containing 10% charcoal-dextran stripped FBS and then added in quadruplicate directly onto cells, both in the presence and absence of 5×10^{-7} M OHF. The A bioactivity measurements were obtained by subtracting the averaged RLUs from quadruplicate wells in the absence of OHF from the averaged RLUs in the presence of OHF per sample. Further, the G bioactivity was obtained from the averaged RLUs from quadruplicate wells in the presence of OHF per sample. The intra-assay and inter-assay CVs of both assays are 4–12%. The minimum detection limit for testosterone mimic and cortisol is 1.25×10^{-10} and 4.4×10^{-9} M, respectively.

3.2.4. Ethical statement

The study was approved by the Institutional Review Boards at the U.S. National Cancer Institute and the Guangdong Poison Control Center in China and all study participants provided written informed consent.

3.2.5. Cell viability assay

Cytotoxicity was evaluated by a quantitative colorimetric PrestoBlue assay following the corresponding procedure described in the steroidogenic gene expression assay. Briefly, NCI-H295R cells were seeded in 96-well microtiter plates (CellStar) at a density of 4.5×10^4 cells/well. Cells were exposed to FA at various concentrations or 0.1% v/v DMSO (vehicle control) and incubated in a humidified incubator at 37°C. After incubation, PrestoBlue reagent was added to each well and cells were incubated for 30 min according to the manufacturer protocol. The plate was measured at the fluorescence of 560 nm (excitation) and of 590 nm (emission) using fluorescent plate reader (BioTek Instruments Inc.).

3.2.6. Steroidogenic gene expression assay

NCI-H295R cells were maintained in DMEM/F12 culture medium containing 10 μM of forskolin for 48 h prior to the chemical exposure as described by Karmaus et al. with minor modifications[13]. Cells were seeded in a 25-cm^2 flask at a density of 3.6×10^6 cells/flask and were exposed to FA at 3 noncytotoxic concentrations: 10^{-6}, 10^{-5}, and 5×10^{-4} M for 48 h. After incubation, RNA extractions were performed.

3.2.7. RNA isolation and real-time quantitative PCR

After a 48 h incubation period with the test chemicals, total RNA was extracted from NCI-H295R cells using RNeasy Mini Kit (Qiagen). Briefly, cell lysates were transferred into a QIAshredder column (Qiagen) and centrifuged for 2 min. Then, the same volume of freshly prepared 70% ethanol was added into the cell lysates. The lysate mixture was purified using the RNeasy Mini Kit with on column DNase digestion. Subsequent to purification, RNA purity and concentration were determined using a Cytation5 spectrophotometer (BioTek Instruments Inc.). The ratio of absorbance at 260/280 greater than 2.1 qualified for cDNA synthesis. Purified RNA was used immediately for reverse transcription or stored at -80°C until further analysis. Total RNA was reverse transcribed to CDNA in a 20 μL reaction mixture using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. The cDNA was amplified in 10 μL of 2 × TaqMan gene expression master mix with the following TaqMan assay primers and probes from Applied Biosystems: StAR (Hs00986559_g1), CYP17A1 (Hs01124136_m1), CYP11A1 (Hs00167984_m1), 3βHSD2 (Hs00605123_m1), 17βHSD1 (Hs00166219_g1), CYP19A1 (Hs00903411_m1), CYP21A1 (Hs01021123_g1), CYP11B1 (Hs01596406_gH), and CYP11B2 (Hs01597732_m1). Actin (ACTB) (Hs99999903_m1) was used as a housekeeping gene to normalize the expression of the target genes. Quantification of the expression of target genes was based on a comparative cycle threshold (Ct) value and normalized to ACTB. The fold change of target genes was compared to the vehicle control using the 2-ΔΔCt method[20].

3.2.8. Statistical analysis

All statistical analyses in the FA study were performed using Statistical Analysis Software (SAS v. 9.1.3, Cary, NC). Previously conducted analyses have been described elsewhere[4, 15]. Briefly, unadjusted means and standard deviations were calculated for all endpoints. Linear regression using the natural logarithm of each endpoint was used to test for differences between workers exposed to FA and control workers. All regression models were adjusted for age (continuous variable). Further adjustment for current cigarette smoking (yes/no), current alcohol consumption (yes/no), recent respiratory infection (yes/no) and body mass index (BMI) was conducted if the regression coefficient of the corresponding exposure variable, was altered by more than 15% change. In the in vitro study, data were expressed as means ± S.E.M. of three independent experiments. Statistical comparisons between each test chemical and controls were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test. Differences with $p<0.05$ were considered statistically significant.

3.3. Results

In the FA study, the exposed group was selected based on high exposure levels (median FA level = 1.3 ppm in air) compared to the unexposed control group. Demographic characteristics including age, alcohol consumption, smoking status, recent infection status and BMI were comparable among the exposed and control groups (Table 1).

There was no significant difference in serum E activity in the FA exposed group (P=0.82) relative to controls, adjusted for age, smoking, alcohol consumption, and recent infection (Figure

1). After adjusting for age and BMI, serum A bioactivity was not affected by FA exposure (P=0.52) (Figure 2). Moreover, there was no significant difference in serum G bioactivity between the FA exposed and control groups (P=0.45) (Figure 3).

NCI-H295R cells express all important steroidogenic genes and undertake steroid biosynthesis. No cytotoxic effects were observed in any of the concentrations of FA (Figure 4). The expression of all steroidogenic genes including StAR, CYP11A1, CYP17A1, 3βHSD2, 17βHSD, CYP19A, CYP21A1, CYP11B1, and CYP11B2 were upregulated in the forskolin exposed group (positive control). There were significant changes in the expression of CYP11A1, CYP17A1, CYP19A, CYP21A1, CYP11B1, and CYP11B2 in the prochloraz exposed group (negative control). There was also no significant change in the expression of steroidogenic genes in response to FA exposure (Figure 5).

3.4. Discussion

In the present study, we did not observe a significant difference in serum E, A, and G bioactivities, supporting previous findings showing that there was no significant change in serum testosterone and estradiol levels in Sprague-Dawley rats exposed to FA[9, 10]. However, in a short-term human study exposed to FA, alterations in serum testosterone and follicle stimulating hormone, but not LH, 17β-estradiol, and prolactin were observed[12]. Discrepancies between studies may be explained by duration and magnitude of exposure as the levels of FA exposure in the previous study were not measured[12].

TaqMan gene expression assays were used to measure steroidogenic gene expression to confirm the findings in the cross-sectional study. Steroidogenesis is series of enzymatic conversions of cholesterol into steroid hormones. This series of biochemical pathways can be affected by chemical exposures. The first step involves cholesterol transport into the mitochondria, which is facilitated by the Steroidogenic Acute Regulatory protein (StAR). CYP11A1 catalyzes the conversion of cholesterol to pregnenolone inside the mitochondria. The final production of estrogens, androgens and glucocorticoids requires additional biochemical conversions involving multiple enzymes such as CYP17A1, 3βHSD2, CYP19A1, CYP21A1, CYP11B1 and CYP11B2. We found that forskolin, a positive control, upregulated the expression of many steroidogenic genes while prochloraz, a negative control, altered the expression of key steroidogenic genes, consistent with other studies[14, 21]. FA did not influence gene expression involved in steroidogenesis at any concentration, showing that FA does not affect steroid hormone production in vitro. These mechanistic, in vitro results support the null observation in the FA epidemiological study that hormone receptor bioactivities were not significantly different between the exposed and the control groups.

Our molecular epidemiological study had some notable limitations. Notably, we had relatively small sample size that lowered statistical power therefore minimizing the chance to detect a true effect of FA exposure on hormone receptor functions. Future studies including a larger number of FA exposed and control workers may be needed to fully investigate the effect of FA exposure on circulating hormone levels, but the data presented here do not suggest a need for such studies. Further, since we did not have measures of endogenous estrogens, androgens and glucocorticoids, which would have enabled us to estimate what proportion of the variability

in hormone receptor bioactivities might be due to differences in the level of exogenous chemicals and the endogenous hormones.

3.5. Conclusion

The present study shows that human FA exposure does not affect hormone receptor bioactivity as there was no alteration in serum E, A, and G activities in FA exposed workers and does not alter steroidogenesis in vitro providing evidence that FA act as an endocrine disruptor in altering estrogen, androgen, and/or glucocorticoid related pathways.

3.6. References

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Figures and tables

Table 1. Demographic characteristics of controls and workers exposed to formaldehyde in a cross-sectional molecular epidemiology study conducted in Guangdong, China.

Characteristics	Formaldehyde	
	Control	Exposed
	n = 44	n = 37
Age, mean (SD) [a]	29.0(7.0)	31.0(6.0)
BMI, mean (SD) [a]	22.1(2.8)	21.6(2.6)
Current alcohol use, n (%)		
Yes	23(52)	26(70)
No	21(48)	11(30)
Current smoke [b], n (%)		
Yes	21(48)	19(51)
No	23(52)	18(49)
Recent infection [b], n (%)		
Yes	29(66)	23(62)
No	15(34)	14(38)
Median exposure, ppm	0.026	1.30

BMI, body mass index.
[a]Mean ± standard deviation.
[b]Number (percent).

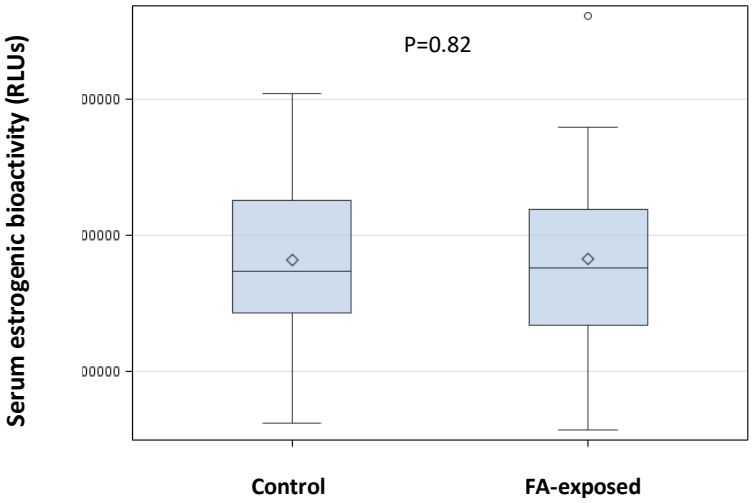

Figure 1. Box and whisker plots, depicting the median (line), lower and upper interquartile range (IQR; box), mean (open square dots) and whiskers to the highest and lowest values of average serum estrogenic bioactivity by formaldehyde (FA) exposure category. P-values between controls and the exposed group for serum estrogenic bioactivity is 0.82. Levels of serum estrogenic bioactivity was adjusted for age, smoking, alcohol consumption, and recent infection status.

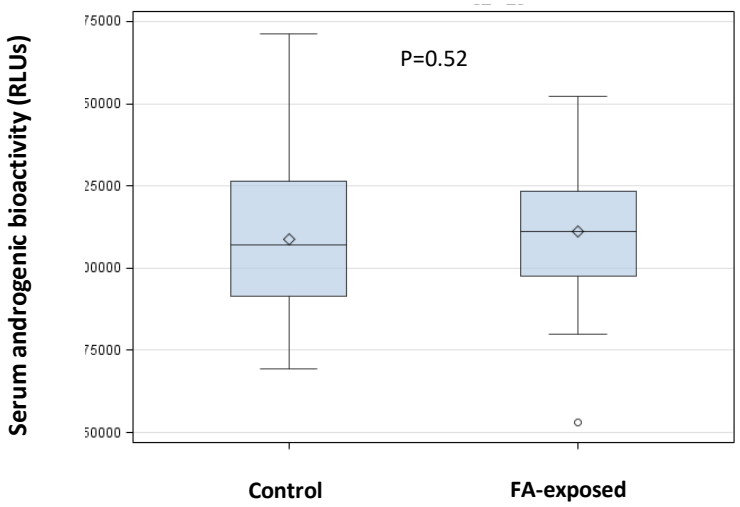

Figure 2. Box and whisker plots, depicting the median (line), lower and upper interquartile range (IQR; box), mean (open square dots) and whiskers to the highest and lowest values of average serum androgenic bioactivity by formaldehyde (FA) exposure category. P-values between controls and the exposed group for serum androgenic bioactivity is 0.52. The serum androgenic bioactivity was adjusted for age and BMI.

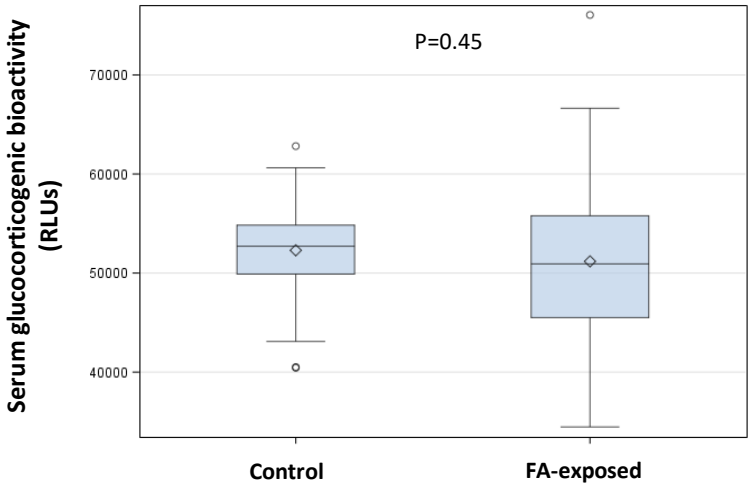

Figure 3. Box and whisker plots, depicting the median (line), lower and upper interquartile range (IQR; box), mean (open square dots) and whiskers to the highest and lowest values of average serum glucocorticogenic bioactivity by formaldehyde (FA) exposure category. P-values between controls and the exposed group for serum glucocorticogenic bioactivity is 0.45. The serum glucocorticogenic bioactivity was adjusted for age.

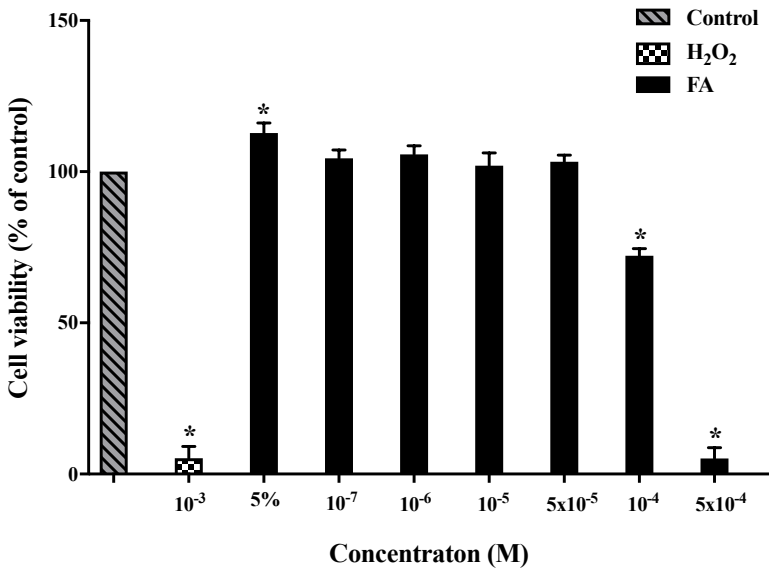

Figure 4. The cytotoxic effect of FA measured by PrestoBlue assay. NCI-H295R cells were exposed to various concentrations of FA ranging from 10^{-7} to 5 x 10^{-5} M, 0.1% v/v DMSO (vehicle control), 5% Nu-serum (positive control), and 10^{-3} M H_2O_2 (negative control) for 48 h. Data are presented as relative percent change compared with 0.1% v/v DMSO. Values are expressed as the mean ± S.E.M. of three independent experiments. * $p < 0.05$ indicates a significant difference between exposed groups and the corresponding control group (0.1% v/v DMSO).

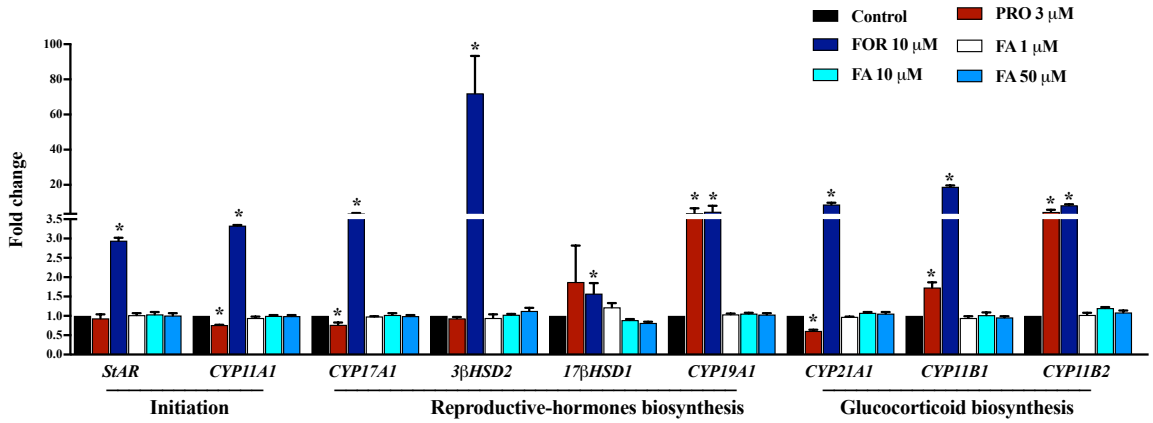

Figure 5. The effects of FA on the expression of genes related to steroidogenesis pathways in NCI-H295R cells. Cells were exposed to FA at concentrations from 1 μM – 50 μM, 0.1% v/v DMSO (vehicle control), 10 μM forskolin (positive control), and 3 μM prochloraz (negative control) for 48 h. Values are expressed as the mean fold change ± SEM of duplicate measurements in at least three independent experiments. * $p < 0.05$ indicates a significant difference between exposure groups and the corresponding control group (0.1% v/v DMSO).

Chapter 4

Plasma Glucocorticogenic Activity Differs by Race/Ethnicity Category and Alcohol Intake Among San Francisco Bay Area Women

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Abstract

Racial and ethnic minorities are at higher risk for a variety of diseases. Stress and its biological consequences have been hypothesized to mediate adverse disease outcomes. While socio-demographic and lifestyle factors contribute to racial/ethnic disparities in various diseases including breast cancer, the biological processes underlying these associations remain poorly understood. In fasting morning samples of 503 control women from the San Francisco Bay Area Breast Cancer Study, we used a sensitive Chemical-Activated LUciferase gene eXpression (CALUX) assay to examine the association between race/ethnicity categories and disease risk factors with glucocorticogenic (G) activity, a measure that reflects total plasma glucocorticoids including cortisol levels. Associations between G activity and socio-demographic and lifestyle factors were examined using multivariable linear regression models. Latina and non-Latina Black (NLB) women had 9% (P = 0.053) and 14% (P = 0.008) lower morning G activity than non-Latina White (NLW) women, respectively. Additionally, we replicated a previously reported association between G activity and alcohol intake (women who drank >10 g had 19% higher G activity than non-drinkers, P = 0.004) in Latina and NLB women. Our results suggest that NLB and Latina women may have a blunted cortisol (the most abundant endogenous glucocorticoid in human plasma) awakening response due to chronic stress. Further research should assess the association between G activity and health outcomes in a prospective cohort so as to characterize the relationship between pre-disease chronic stress and disease outcomes across different racial/ethnic populations.

4.1. Introduction

Minority populations in the U.S. are at higher risk for a variety of diseases including high blood pressure [1], cardiovascular disease [2], and cancers [3, 4] and tend to have worse outcomes than non-Latino Whites (NLWs) [5, 6]. As minorities generally have lower levels of socioeconomic status (SES) and have higher levels of psychosocial stress and stressor exposure than NLWs [7, 8], stress may be a possible pathway by which minorities incur greater disease burden. One emerging field of interest is the role of stress in cancer etiology [9, 10]; however, the empirical findings have been inconsistent. For example, a study by Lillberg et al. reported an elevated risk of breast cancer in relation to past stressful life events in a cohort study of 10,808 women in Finland [11]. Women who developed breast cancer were more likely to have severely threatening life experiences than the women without breast cancer [12, 13]. However, other studies found no association between stress and breast cancer risk [14]. One mechanistic explanation for stressors influencing health disparities is through stress-related alterations in biological response systems. Exposure to stressors has an impact on the body's biology as a result of perturbations to the hypothalamic-pituitary-adrenal (HPA) axis, resulting in alterations in the production and secretion of glucocorticoids from the adrenal cortex [15]. Cortisol, the most abundant endogenous glucocorticoid, plays a prominent role in maintaining homeostasis in response to metabolism changes and stressful perturbations [15]. Previous studies have reported variation in diurnal cortisol levels by race/ethnicity category [16, 17].

The biological and physiological actions of glucocorticoids are exerted through the glucocorticoid receptor (GR), which belongs to the classical nuclear receptor superfamily of ligand-dependent transcription factors. Upon glucocorticoid binding, the GR initiates the transcription of target genes in response to stress stimuli. Apart from endogenous glucocorticoids, exogenous chemicals or environmental stressors, like endocrine disruptors that modulate the glucocorticogenic (G) signaling pathway, have been found to be associated with adverse health outcomes. For example, bisphenol A (BPA), which is ubiquitously found in polycarbonate plastics and epoxy resins, has been found to bind GR in vitro and in silico with a similar binding interaction to cortisol and dexamethasone, suggesting an agonistic effect on GR [18]. Furthermore, epidemiological and in vivo studies revealed that BPA exposure is associated with anxiety and depression [19, 20]. Voisin et al., found that a cholesterol metabolite, 6-oxo-cholestan-3β,5α-diol (OCDO), induces breast cancer proliferation through GR activation and the proliferative effect of OCDO was completely attenuated by a GR inhibitor [21]. These studies not only emphasize the important role of glucocorticoids in disease pathologies, but also provide evidence that both endogenous and exogenous chemicals that modulate the GR have an impact on health.

Many studies have investigated the association between stress and adverse health outcomes by measuring cortisol in biological specimens like saliva [22], hair follicles [23], and plasma [24]. These methods lack information regarding the biological activity and the combined effects of glucocorticoids and G compounds present in biological specimens. As a result, novel methods that simultaneously capture both endogenous and exogenous chemicals modulating the GR signaling pathway may provide a better assessment than measuring cortisol levels alone.

Chemical-Activated LUciferase gene eXpression (CALUX) assays have been widely utilized in epidemiologic research to provide an insight into the biological response to chemical exposure in humans [25, 26]. Luciferase assays measure the summated biological activity of all agonists and antagonists on hormone receptors [26, 27].

In a small study of foreign-born and U.S.-born Mexican women (n=90), we found that plasma G activity was associated with alcohol intake [28]. In the present study, we investigated the plasma G activity, which captures all endogenous and exogenous G compounds, in fasting morning samples of women who participated as controls in the San Francisco Bay Area Breast Cancer Study (SFBCS). We examined the association between plasma G activity, demographic and lifestyle factors in NLW, non-Latina Black (NLB), and Latina women.

4.2. Materials and Methods

4.2.1. Sample collection and procedure

Participants in the present study were selected from the control group of the SFBCS, which is a multiethnic population-based case-control study of breast cancer as described elsewhere [29]. The SFBCS controls were identified by random-digit dialing method between 1996 and 2001. Trained professional interviewers administered questionnaires in English or Spanish at a home visit and took anthropometric measurements. A short questionnaire was administered at the time of blood draw in order to update some important variables. In the present study, plasma G activity was measured from 503 women, including 74 NLW, 100 NLB, and 329 Latina women. The age range of the study participants was between 35 to 79 years.

4.2.2. Plasma Glucocorticogenic Activity Measurement

To measure plasma G activity, we used a GR mediated CALUX assay, which allowed us to capture total effects of both endogenous and exogenous G compounds. We used procedures as described in the previous study with minor modifications [28]. In short, the MDA-kb2 cell line (ATCC®CRL-2713™), a human triple negative breast cancer cell line stably transfected with the murine mammalian tumor virus (MMTV) luciferase neo reporter gene construct, was obtained from the American Type Culture Collection (ATCC). These cells highly express both endogenous androgen receptor and GR, which stimulate the MMTV promoter. To measure G activity in plasma, we used a potent androgen receptor inhibitor, hydroxyflutamide (Sigma-Aldrich, St. Louis, USA). MDA-kb2 cells were routinely maintained in Leibovitz's-15 (L-15) medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Corning, Bedford, MA, USA) at 37°C in a humidified incubator with no CO_2. Cell culture media were changed 3 times per week. External sources of total steroids were removed by maintaining the cells with L-15 medium supplemented with 10% charcoal-dextran stripped FBS for 1 week prior to the luciferase assay. The cells were then seeded at a density of 2.7×10^4 cells/well in white, 96-well microtiter plates (Thermo Scientific, Grand Island, NY, USA) and incubated at 37°C for 24 hours. After this initial incubation period, the 80 µL of fasting morning plasma used per female sample was diluted in L-15 medium containing 10% charcoal-dextran stripped FBS and then added in triplicate directly onto cells, in the presence of 5×10^{-7} M hydroxyflutamide. After a second incubation of 24 hours, the cells were lysed with 1× passive

lysis buffer (Promega, Madison, WI, USA) and the microplate was read using a luminometer (Berthold Technologies, Oak Ridge, TN, USA). Readings from each well were reported in relative light units (RLUs) with higher RLU values indicating greater plasma G activity. The RLUs from triplicate wells were averaged to achieve one measurement of G activity per individual. The overall intra-assay and inter-assay coefficients of variation of this assay were 7–12%. The minimum detection limit for cortisol was 4.4 nM.

4.2.3. Ethical statement

The study was approved by the Institutional Review Boards at the University of California, San Francisco and the Cancer Prevention Institute of California and all study participants provided written informed consent.

4.2.4. Statistical analysis

Differences in means and proportions for analyzed variables between racial/ethnic categories were assessed by two-tailed t-tests and Fisher's exact tests, respectively. Averaged RLUs from the luciferase assay were natural log (ln) transformed in order to approximate normal distribution. Plate adjusted values were obtained by first estimating average plate effects using linear regression and then subtracting the average plate effect from each individual value. A linear regression analysis was used to analyze the relationship between ln-transformed and plate adjusted G activity as the outcome, with race/ethnicity category, socio-demographic, and lifestyle factors as predictors. Percent change in RLUs per unit change of predictor variables was calculated using the formula $[e^{\beta}-1]*100$. The cut off for statistical significance was set at a p value of 0.05 or less. All analyses were performed using Stata or R. Multivariable regression models included race/ethnicity category (Latina, NLW, NLB), age at blood draw (categorical, <55; 55-65; >65 years), height (continuous, cm), body mass index (BMI: <25 kg/m^2; 25 to <30 kg/m^2; ≥30 kg/m^2) at interview, neighborhood SES (categorical with values 1 to 5, 1=lowest SES, 5=highest SES) [30], based on Census 2000 tract-level data, alcohol intake at interview (categorical, None; <10gms daily; 10 or >gms daily) as predictors, and plasma G activity (continuous, ln-transformed) as the outcome variable. Participants who had missing variable information were removed from the analyses. We also conducted three additional analyses stratifying by racial/ethnic category. A subset of Latina women (n=279) was used in a separate analysis of Indigenous American (IA) ancestry, socio-demographic, and lifestyle factors. The proportion of IA ancestry was used as a continuous variable ranging from 0 to 1. The multivariable model for the ancestry analysis included IA ancestry, age at blood draw, height, BMI, neighborhood SES, alcohol consumption and nativity (foreign-born vs. U.S.-born). In addition, subsets of NLW (n=72) and NLB (n=99) women were used in the analysis of socio-demographic and lifestyle factors. The multivariable model for the socio-demographic and lifestyle factors analysis included age at blood draw, height, BMI, neighborhood SES, and alcohol consumption.

4.3. Results

4.3.1. Baseline characteristics

In the present study, we investigated if individuals from different racial/ethnic groups (NLWs, NLBs, and Latinas) had different plasma G activity as measured by GR mediated luciferase reporter gene assay. Furthermore, we evaluated if plasma G activity was associated with other factors such as age, alcohol intake, BMI, and SES. Demographic characteristics by racial/ethnic category are presented in Table 1. Overall, there was a statistically significant difference in the level of GR RLUs between racial/ethnic categories. G activity was highest among NLW women (mean RLUs of 48845), followed by NLBs (mean RLUs of 43561), and lowest among Latina women (mean RLUs of 42776). Other factors such as age at blood draw, height, place of birth, alcohol intake, BMI, and SES were also significantly different between groups. For example, Latina women were younger at blood draw (61 yrs) compared to NLB (62 yrs) and NLW (67 yrs) women. The majority (66%) of the Latina women were foreign-born, while 92% and 97% of the NLW and NLB women were U.S.-born, respectively.

4.3.2. Plasma glucocorticogenic activity

Plasma G activity was obtained for 503 participants. In the univariable analysis, we found NLB and Latina women had 11% (P = 0.018) and 13% (P = 0.001) lower plasma G activity compared to NLWs, respectively (Table 2). In the multivariable analysis, we still observed a significantly lower plasma G activity among Latinas (9%, P = 0.053) and NLBs (14%, P = 0.008) than NLWs. We did not observe a linear trend between increasing age and plasma G activity. However, when compared to women who were younger than 55 years, women who were between 55 to 65 years of age had lower G activity (21%, p=0.016), but the association was not significant in women who were older than 65 years. We observed a positive association between G activity and height. Moreover, women who reported drinking more than 10 g of alcohol daily had 19% (P = 0.004) higher plasma G activity compared to non-drinkers. The association between G activity and alcohol intake was not observed in women who drank less than 10 g/day (Table 2).

4.3.3. Subgroup analyses

Latina women who had available ancestry information were included in a subgroup analysis to determine the association between plasma G activity, IA ancestry, and other factors. We found plasma G activity was inversely associated with IA ancestry. This inverse association was statistically significant in the univariable analysis (25% change in plasma G activity, P = 0.026) and marginally significant in the multivariable analysis (22% change in plasma G activity, P = 0.076) (Table 3). When compared to women who were younger than 55 years, women who were between 55 to 65 years had lower G activity (11 %, P = 0.015). The association between plasma G activity and alcohol intake was observed only in women who drank more than 10 gms, with higher G activity in this group compared to nondrinkers (27 %, P = 0.021). No other variables in the model were statistically significantly associated with G activity.
NLW and NLB women were included in a separate subset analysis to determine the association between plasma G activity and other factors. We observed a suggestive difference in plasma G

activity in NLB women who drank more than 10 gms compared to non-drinkers (23% change in G activity, P = 0.09) (Table 4). However, this association was not observed in NLW women (Table 5).

4.4. Discussion

The results of the present study suggest that plasma G activity varies by racial/ethnic category in the U.S., with lower activity among NLB and Latina women relative to NLW women. In addition, we replicated a previously reported positive association between G activity and alcohol intake [28] and found that among Latina women, those with higher IA ancestry had a lower average level of plasma G activity.

It has been suggested that stress-related disturbance in the HPA axis, especially cortisol regulation, may influence adverse health outcomes. Recent evidence indicates flatter diurnal rhythms (e.g., due to lower morning and/or elevated evening cortisol levels) among people having stressful life events, difficulty in personal relationships, and trauma [31-33]. Furthermore, flatter diurnal cortisol slopes have also been implicated in the etiology of a variety of disease outcomes such as breast cancer mortality and diabetes [34, 35]. Sephton et al. found that cortisol slopes can be used as a predictor for breast cancer survival [35]. The study showed that breast cancer patients with earlier mortality had flatter diurnal cortisol slopes which indicates a lack of normal cortisol rhythms [35]. Although NLB and Latina women have a lower incidence of breast cancer compared to NLW women, they tend to have a higher risk of mortality from the disease [36, 37]. The significantly lower plasma G activity shown among NLB and Latina when compared to NLWs in this study may be due to blunted cortisol awakening response potentially due to chronic stress, which could explain observed disparities in disease outcome such as a poorer survival rate for breast cancer when compared to NLW women.

Studies have shown that socio-demographic and lifestyle factors like SES and alcohol consumption are associated with cortisol levels [38]. We found that plasma G activity was associated with alcohol consumption. Women who reported drinking more than 10 g of alcohol daily had higher plasma G activity compared to non-drinkers, which is in an agreement with previous findings [39, 40]. In a study of men and women, alcoholics had about three times greater hair cortisol levels than abstinent alcoholics or non-alcoholics [39]. Abstinent and non-alcoholics had similar hair cortisol levels, indicating that cortisol levels reduce to baseline levels after extended alcohol cessation [39]. Thayer et al., reported that healthy men in the highest tertile of self-reported alcohol intake had greater urinary cortisol levels compared to men in the lower tertiles of alcohol intake [40]. Furthermore, these findings support previous studies on the HPA axis dysregulation in heavy drinkers and suggest impaired negative-feedback mechanism of the HPA axis in heavy drinkers [41, 42]. Another mechanism that explains the observed association between alcohol consumption and G activity is the activation of the GR. Inhibition of GR function reduces motives for drinking alcohol, which suggests that higher G activity might lead to higher motivation for alcohol intake [43].

We observed the inverse association between plasma G activity and IA ancestry among Latina women which may be explained by environmental factors such as exposure to exogenous compounds or dietary constituents [44]. Although, younger generations acculturate to the U.S.

lifestyle, they may retain their cultural diet. A previous in vitro study has shown that two phytochemicals in beans, genistein and daidzein, directly suppress adrenocortical steroidogenesis through the enzymatic inhibition of 3β-hydroxysteroid dehydrogenase and cytochrome P-450 21-hydroxylase and in turn decrease cortisol production [45]. In animal studies, administration of genistein decreases plasma adrenocorticotropic hormone (ATCH) and corticosterone (the most abundant glucocorticoid in rodents) [46, 47]. Furthermore, daidzein administration suppresses ACTH and corticosterone secretion in Wistar rats [48]. Another explanation could be a genetic difference by ancestral component in lipoprotein and cholesterol metabolism, as some cholesterol metabolites have been shown to affect the GR signaling pathway [21, 49, 50]. However, these hypotheses warrant further investigation.

There were some limitations that are worth noting. Our study does not account for measures of endogenous cortisol levels, which would have allowed us to calculate the proportion of plasma G activity that might result from differences in the level of exogenous compounds versus endogenous cortisol levels. However, we were able to measure summated G activity which reflects biological activity of glucocorticoid-like compounds of endogenous and exogenous origins, therefore providing a comprehensive representation of total G exposure. Another limitation is that our study had a single time point measurement of plasma G activity and therefore we did not know diurnal cortisol rhythms for each individual. Moreover, we lacked information on the profile of other hormones in the HPA axis that would have allowed us to gain more insight into the whole HPA axis.

4.5. Conclusion

The present study reports differences in plasma G activity among racial/ethnic categories, which may provide useful insight into a mechanistic explanation for the wide spectrum of health disparities in different population groups. We also replicated the previously reported positive association between G activity and alcohol intake. Although we could not identify the specific glucocorticoid-like compounds that are acting on the GR, we provide useful information on the association between biological activity of GR, racial/ethnic categories and lifestyle/demographic factors. Advanced mass spectrometry-based technology is needed to identify the specific compounds contributing to the observed associations. In addition to the traditional measurements of cortisol as a biomarker of stress, we highlight that the plasma G activity can be utilized in epidemiologic research as an alternative biomarker that can simultaneously provide insight into biological activity of endogenous glucocorticoids and glucocorticoid-like compounds. Using measurements of G activity can be a useful tool aimed at addressing differences in health disparities among different population groups, particularly in populations that are often exposed to more stressors and experience worse disease outcomes.

4.6. References

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Tables

Table 1. Demographic characteristics of the study subjects by different racial/ethnic categories.

Continuous variables	Latinas			NLW			NLB			P value
	N	Mean	Sd.	N	Mean	Sd.	N	Mean	Sd.	
GR RLUs plate adjusted	329	42776	15182	74	48845	17157	100	43561	15555	0.0056
Age at blood draw, yrs	329	61.49	9.5	74	66.8	10.5	100	61.66	9.9	0.0001
Height, cm	324	155.47	6.94	73	161.14	7.34	100	163.31	6.25	<0.0001
Individual African Ancestry proportion (mean %)	285	0.08	0.07							
Individual European Ancestry proportion (mean %)	285	0.51	0.15							
Individual IA ancestry proportion (mean %)	285	0.41	0.15							
Categorical variables	**Latinas**			**NLW**			**NLB**			**P value**
	N	%		N	%		N	%		
Place of birth										
US-born	112	34		68	92		97	97		<0.0001
Foreign-born	217	66		6	8		3	3		
Alcohol Intake per day (g/day)										
None	235	71		34	46		76	76		<0.0001
<10	82	25		20	27		15	15		
≥10	12	4		20	27		9	9		
BMI (kg/m^2)										
<25	42	13		22	30		16	16		<0.0001
25 to <30	122	38		30	41		24	24		
≥30	160	49		21	29		60	60		
Age (years)										
<55	94	29		14	19		31	31		0.001
55-65	117	36		13	18		31	31		
>65	118	36		47	64		38	38		
Neighborhood Socioeconomic Status										
1 (low SES)	17	5		3	4		23	23		<0.0001
2	77	24		6	8		30	30		
3	91	28		12	16		25	25		
4	80	25		21	29		11	11		
5 (high SES)	61	19		31	42		10	10		

Table 2. Association between plasma glucocorticogenic activity, lifestyle, and demographic factors (N=492).

Univariable analysis	Coefficient	P value
Race/ethnicity		
NLW	ref	
NLB	-0.12 (-0.22, -0.02)	0.018
Latina	-0.14 (-0.22, -0.05)	0.001
Multivariable analysis	**Coefficient**	**P value**
Race/ethnicity		
NLW	ref	
NLB	-0.15 (-0.26, -0.04)	0.008
Latina	-0.09 (-0.19, -0.00)	0.053
Age (years)		
<55	ref	
55-65	-0.09 (-0.17, -0.02)	0.016
>65	-0.03 (-0.10, 0.05)	0.454
Height, per 10 cm	0.05 (0.00, 0.09)	0.037
BMI (kg/m^2)		
<25	ref	
25 to <30	0.01 (-0.07, 0.10)	0.752
≥30	0.04 (-0.05, 0.12)	0.407
Neighborhood SES		
1 (low SES)	ref	
2	-0.04 (-0.16, 0.08)	0.482
3	-0.05 (-0.17, 0.07)	0.418
4	-0.11 (-0.23, 0.01)	0.079
5 (high SES)	-0.11 (-0.24, 0.01)	0.086
Alcohol intake per day (g/day)		
None	ref	
<10	0.01 (-0.06, 0.09)	0.677
≥10	0.17 (0.07, 0.29)	0.004

Table 3. Association between plasma glucocorticogenic activity, lifestyle, and demographic factors in Latina women (N=279).

Univariable analysis	Coefficient	P value
Indigenous American ancestry	-0.29 (-0.55, -0.03)	0.026
Multivariable analysis	**Coefficient**	**P value**
Indigenous American ancestry	-0.25 (-0.53, 0.03)	0.076
Age (years)		
<55	ref	
55-65	-0.12 (-0.23, -0.02)	0.015
>65	-0.03 (-0.13, 0.07)	0.59
Height, per 10 cm	0.06 (0.00, 0.13)	0.061
BMI (kg/m^2)		
<25	ref	
25 to <30	0.02 (-0.11, 0.15)	0.765
≥30	0.04 (-0.08, 0.17)	0.49
SES		
1 (low SES)	ref	
2	-0.06 (-0.25, 0.12)	0.497
3	-0.08 (-0.27, 0.10)	0.376
4	-0.13 (-0.32, 0.05)	0.156
5 (high SES)	-0.14 (-0.33, 0.06)	0.168
Alcohol intake per day (g/day)		
None	ref	
<10	-0.00 (-0.10, 0.09)	0.928
≥10	0.24 (0.03, 0.44)	0.021
Foreign-born		
Yes	-0.06 (-0.15, 0.03)	0.192

Table 4. Association between plasma glucocorticogenic activity, lifestyle, and demographic factors in non-Latina Black women (N=99).

Multivariable analysis	Coefficient	P value
Age (years)		
<55	ref	
55-65	0.01 (-0.17, 0.18)	0.949
>65	-0.05 (-0.22, 0.13)	0.61
Height, per 10 cm	-0.13 (-0.25, -0.01)	0.037
BMI (kg/m^2)		
<25	ref	
25 to <30	0.01 (-0.22, 0.24)	0.923
≥30	-0.02 (-0.22, 0.17)	0.813
Neighborhood SES		
1 (low SES)	ref	
2	-0.05 (-0.25, 0.14)	0.6
3	-0.01 (-0.22, 0.19)	0.896
4	0.01 (-0.25, 0.27)	0.941
5 (high SES0	-0.16 (-0.46, 0.13)	0.266
Alcohol intake per day (g/day)		
None	ref	
<10	0.17 (-0.04, 0.38)	0.12
≥10	0.21 (-0.04, 0.47)	0.094

Table 5. Association between plasma glucocorticogenic activity, lifestyle, and demographic factors in non-Latina White women (N=72).

Multivariable analysis	Coefficient	P value
Age (years)		
<55	ref	
55-65	-0.22 (-0.46, 0.03)	0.078
>65	-0.09 (-0.30, 0.12)	0.4
Height, per 10 cm	0.08 (-0.03, 0.19)	0.135
BMI (kg/m^2)		
<25	ref	
25 to <30	-0.03 (-0.22, 0.15)	0.732
≥30	0.06 (-0.14, 0.26)	0.553
Neighborhood SES		
1 (low SES)	ref	
2	0.01 (-0.46, 0.48)	0.977
3	-0.13 (-0.56, 0.29)	0.534
4	-0.26 (-0.67, 0.15)	0.211
5 (high SES)	-0.33 (-0.72, 0.07)	0.102
Alcohol intake per day (g/day)		
None	ref	
<10	-0.07 (-0.25, 0.12)	0.475
≥10	0.05 (-0.14, 0.24)	0.581

Chapter 5

Conclusions

5.1. Summary of major findings

The dissertation objective was to assess the effects of chemical exposure and other factors including race/ethnicity, alcohol consumption, and Indigenous American ancestry on receptor hormone bioactivities in human biospecimens.

The following are the main findings from each research project:

Trichloroethylene exposure is associated with increased serum estrogenic, but not androgenic bioactivities in exposed workers.

Chapter 2 provides support for an alternative method to assess the impact of chemical exposure on the endocrine system. Chemical-Activated LUciferase gene eXpression (CALUX) assays were used to measure serum hormone receptor bioactivity in workers exposed to industrial chemicals. Previous epidemiologic research investigating the effects of trichloroethylene (TCE) exposure on the endocrine system has notable limitations, including flawed exposure assessments and potential confounding by other exposures. This dissertation study addressed these limitations by conducting a cross-sectional molecular epidemiological study of male workers exposed to TCE using personal air measurement and excluded factories that used other chemicals that might act as potential confounders. This study is the first to report that, by altering estrogenic activity, TCE is a potential endocrine disruptor in humans. Moreover, we found that elevated estrogenic bioactivity levels were significantly associated with haemato-immunological parameters such as white blood cell count, granulocyte counts, and monocyte count No disruption in androgenic bioactivity was observed.

Formaldehyde exposure did not affect serum estrogenic, androgenic, and glucocorticogenic bioactivities.

In Chapter 3, we conducted a cross-sectional molecular epidemiological study of male workers occupationally exposed to formaldehyde (FA) in China. Data from previous studies that assessed the effects of FA exposure on hormone levels were inconsistent. CALUX assays were used to measure hormone receptor bioactivities in FA exposed workers and unexposed controls. We found no alteration in estrogenic, androgenic, and glucocorticogenic bioactivities in exposed workers, providing evidence that FA exposure does not alter serum estrogen, androgen, and glucocorticoid levels.

Racial/ethnic difference, alcohol consumption, and Indigenous American Ancestry influence plasma glucocorticogenic bioactivity.

Chapter 4 demonstrated the effects of various factors including race/ethnicity, Indigenous American ancestry, and alcohol intake on plasm glucocorticogenic bioactivity. We found significantly lower plasma G activity among Non-Latina Black (NLB) and Latina women

relative to Non-Latina White (NLW) women. This finding suggests that NLB and Latina women may have blunted cortisol awakening response due to chronic stress.
This finding could explain health disparities in adverse health outcomes such as poorer survival rates of breast cancer among NLB and Latina women. Moreover, we also found that women who drank alcohol more than 10 g/day had higher plasma glucocortocogenic activity than non-drinkers which substantiate previous reports that heavy alcohol consumption disrupts the hypothalamic-pituitary-adrenal (HPA) axis and negative-feedback mechanism of the HPA axis. There was no previous study examining the association between plasma glucocorticoids and Indigenous American ancestry in Latina women. We found a borderline significant difference between plasma glucocorticogenic activity and Indigenous American ancestry, which warrants future studies using a larger number of Latinas.

5.2. Recommendations

Based on the findings reported in this dissertation study I recommend the following areas for additional research.

Endogenous hormone measurements

Because the CALUX assay detects total biological activity of all endogenous and exogenous compounds acting on hormone receptors, it cannot identify specific chemicals that produce hormone bioactivity. Thus, future work should employ advanced analytical platforms, particularly mass spectrometers, to identify the exogenous and/or endogenous chemicals that produced the observed associations.

Health effects of TCE at low concentrations

According to the Occupational Safety and Health Administration (OSHA), the permissible exposure limit (PEL) of TCE exposure in the workplace is currently 100 ppm. The levels of TCE in workplace air in this study were much lower than this PEL (13.9 ppm). Yet, we observed a significant disruption in serum estrogenic levels in the exposed workers. Thus, the current PEL of 100 ppm may not be sufficient to protect workers from adverse effects of TCE on the endocrine system. Future studies should comprehensively investigate the effects of TCE exposure at low concentrations on endocrine-related outcomes, including infertility, testicular cancer, and prostate cancer.