ABSTRACT

GREEN, ADRIAN JAMES. Developmental Cadmium Exposure Negatively Impacts Behavior, Osteogenesis, and Adipogenesis *In Vivo*, and *In Vitro* (Under the direction of Dr. Antonio Planchart).

Cadmium (Cd), is a ubiquitous environmental pollutant that affects human populations worldwide primarily through cigarette smoke and the consumption of contaminated foods. Accumulation of Cd has been associated with bone marrow adiposity, osteotoxicity, and balance and vestibular dysfunction in adults, but little is known about the developmental effects of low concentration Cd exposures. The goal of the research presented herein was to examine the ways that developmental Cd exposure can induce vestibular dysfunction, decrease bone development, and increase adiposity and elucidate the mechanisms through which Cd promotes developmental toxicity. We first investigated the effects of developmental cadmium exposure on body weight and abdominal lipid accumulation in children and juvenile zebrafish, respectively. Our results indicate that the presence of Cd in maternal blood during pregnancy is associated with a ~25-fold increase in obesity odds at age five for every one ng/g increase in blood weight of Cd in the offspring. Zebrafish radioisotope uptake studies with ^{109}Cd revealed that exposure to 60 parts per billion (ppb) Cd resulted in an internal exposure level approximating those observed in maternal blood. This exposure concentration, in a zebrafish model, recapitulated our findings that Cd exposure was associated with increased juvenile adiposity. To understand the mechanisms behind the observed increase in juvenile adiposity, we hypothesized that Cd exposure disrupts mesenchymal stem cell differentiation resulting in bone marrow adiposity, increased lipid accumulation, and osteotoxic effects *in vitro* and *in vivo*. Human adipose-derived mesenchymal stem cells (hADMSCs) were exposed to 0.5 or 5 ppb Cd during adipogenic differentiation. *In vitro* osteotoxicity was assessed in zebrafish embryos exposed to 0 – 100 ppb Cd from four hours

post-fertilization (hpf) through 14 days post-fertilization (dpf). Cd exposure increased the number of hADMSCs that differentiate into adipocytes and significantly increased lipid accumulation. Additionally, at eight dpf zebrafish exposed to 15 ppb Cd had decreased mineralization of the notochord, pharyngeal teeth, and cleithrum compared to unexposed controls while those individuals exposed to 40 ppb Cd showed no evidence of mineralization. At 14 dpf the notochord, pharyngeal teeth, cleithrum, and opercle showed reduced mineralization compared to controls in individuals exposed to Cd at 25 ppb or higher. These data support the hypothesis that developmental exposure to environmentally relevant concentrations of Cd decreases bone mineralization and increases adiposity by disrupting the differentiation of MSCs. Next, considering that Cd is a known ototoxicant we hypothesized that developmental cadmium exposure induces vestibular disruption resulting in behavioral alterations and increased sensitivity to stimuli. Zebrafish embryos exposed developmentally to 10 - 60 ppb Cd from four hpf to seven dpf exhibit abnormal behaviors, including pronounced increases in auditory sensitivity and circling motions, both of which are linked to reductions in otolith growth. Pharmacological intervention shows that agonist-induced activation of the P2X receptor family in the presence of Cd can restore otolith size and minimize behavioral abnormalities. In conclusion, Cd-induced ototoxicity is linked to vestibular-based behavioral abnormalities and auditory sensitivity following developmental exposure. Together these studies highlight that environmentally relevant low concentrations of Cd can have significant impacts on multiple organ systems in a developing organism, and provides important insights into the ways that Cd influences cell fate and induces metabolic and vestibular dysfunction.

Developmental Cadmium Exposure Negatively Impacts Behavior, Osteogenesis, and Adipogenesis *In Vivo*, and *In Vitro*

by
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DEDICATION

This dissertation is dedicated to my amazing wife, whose support, and infinite patience have been invaluable, and to my loving mother who never stopped encouraging me, even when the psychologist said that with dyslexia, I would never graduate eighth grade.

BIOGRAPHY

Adrian J. Green was born March 7th, 1982 to Errol Green and Alison Farrell in Grahamstown in the Republic of South Africa. Growing up Adrian had a passion for biology, computers, and the outdoors, which began at a young age thanks to his mother taking him to lectures as an infant and his parents discussing pharmacy and medicine around the dinner table. Adrian excelled in biology, science, and accounting classes throughout school, and graduated from Parel Vallei High School in Somerset West in 2000. Adrian decided computer science would be a smashing career with the rapid rise of the internet, and therefore graduated with a computer science diploma in 2002. Following graduation Adrian moved to London in the United Kingdom to take a position as a junior programmer and webmaster for software developer SolarSys. During the next two years the ripple effect of the dot com bubble bursting resulted in Adrian deciding to continue his education at Stellenbosch University before transferring to Yuba Community College (YCC) in northern California. While at YCC Adrian rediscovered his passion for biological sciences and went on to attend California State University, Chico where he majored in Microbiology (B.S.), and minored in Chemistry. As an undergraduate Adrian completed an honors research project in environmental microbiology, studying the environmental conditions that promoted the germination of fungal spores. This led Adrian to broaden his research skills by pursuing a master's degree at the University of California, Davis investigating how gestational flame-retardant exposure affects the development of type 2 diabetes mellitus in rats. Adrian successfully earned his Master of Science degree in 2015 and decided to pursue his doctoral degree at North Carolina State University. His interests in developmental toxicology and metabolic dysfunction led him to join the laboratory of Antonio Planchart and Carolyn Mattingly in 2015, where he began his doctoral dissertation research.

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CHAPTER 1

General Introduction

CADMIUM EXPOSURE AND TOXICITY

There is an extensive body of evidence showing that environmental exposure to cadmium results in kidney and bone toxicity following oral exposure, and kidney and lung toxicity following inhalation exposure. The Department of Health and Human Services and the International Agency for Research on Cancer (IARC)[1] have classified cadmium as a probable human carcinogen.

In the United States exposure to cadmium occurs through ingestion of food and water, inhalation of particulates from ambient air or tobacco smoke, and ingestion of contaminated soil or dust[2]. For nonsmokers, food is the primary source of cadmium exposure[3], while inhalation of cigarette smoke is the primary source for smokers[4]. Cadmium incorporates into food through agricultural soils that contain cadmium naturally, from release during mining and other industrial activities that result in atmospheric deposition, or through direct application methods such as phosphate fertilizer application and municipal waste composting[5,6]. Cadmium levels in soils alone are not the only gauge of cadmium concentration in food because other factors, such as the type of crop and farming methods used, are also important[6]. Finally, cadmium-plated utensils and galvanized equipment used in food processing and preparation, enamel and pottery glazes with cadmium-based pigments, and stabilizers utilized in food-contact plastics are also sources of food contamination[7].

Water or ambient air are not major sources of cadmium exposure unless an individual is living in the vicinity of cadmium-emitting industries[8]. The EPA requires that the cadmium

concentration in drinking water be less than five ng/g [2] and water suppliers use hydroxide or carbonate compounds to precipitate and remove cadmium from wastewater and sewage. In a report from IARC[1], these exposures result in an estimated total body burden of non-occupationally exposed adults ranging from 158 to 667 ng/g of body weight in the United States (U.S.) and Europe. Epidemiological studies published since 2015 show that in the U.S the range has declined slightly to 47 to 775 ng/g of body weight based on the Kjellström and Nordberg[9] toxicokinetic model for cadmium.

Children, like adults, are most likely to be exposed to cadmium from the ingestion of food[10] and a study performed in Cincinnati, Ohio, investigated cadmium in human milk and found a mean concentration of 19 ppb[11]. The placenta may act as a partial barrier to fetal exposure as cadmium concentrations have been found to be approximately half as high in cord blood as in maternal blood in several studies including both smoking and nonsmoking women[12–14].

Studies in animals support kidneys, bone, and lungs as sensitive targets of Cd toxicity and provide some suggestive evidence that the developing organisms may also be a target[2]. Other effects observed in humans or animals include reproductive toxicity, hepatic effects, hematological effects, immunological effects, and ototoxicity[2,10,15]. These toxicities are believed to be the result of free cadmium ions that may inactivate metal-dependent enzymes, activate calmodulin, and/or damage cell membranes through production of reactive oxygen species[2,6]. There is a dearth of human data on the developmental effects following exposure to cadmium but some studies indicate that maternal cadmium exposure may cause decreased birth weight in humans[16]. In animals, cadmium has been shown to be a developmental toxicant by inhalation, oral, and parenteral routes resulting in decreased fetal weight, skeletal malformations, and

delayed ossification due to placental toxicity, interference with fetal metabolism, and damage to the maternal liver[2]. Neurodevelopmental effects have been observed at lower concentrations, such as impaired performance in the offspring of rats exposed to cadmium[17].

The mechanisms of cadmium-induced toxicity are not fully understood due to the fact that cadmium can influence a multitude of cellular processes including cell proliferation and differentiation, cell cycle progression, DNA synthesis and methylation, ubiquitination, apoptosis, reactive oxygen species (ROS) production and other cellular activities all of which can potentially lead to cell death[18,19]. Therefore, the mechanisms responsible for cadmium toxicity are likely multifaceted and may vary within different cells and organelles. Upon absorption into the body, cadmium is capable of displacing several different protein-bound essential divalent metals including Zn, Fe, Mg, Mn, Ca, and Se[20] by interfering with coordinate covalent and ionic bonds to sulfur, oxygen, and hydrogen; this likely promotes significant disruption of protein activity required for homeostasis[21]. Therefore, cadmium toxicity may be caused by differences in availability of specific protein binding sites created by developmental or epigenetic variation in the intracellular composition of cells and interference with a diverse array of cell signaling pathways, thereby influencing the activity of receptors, second messengers, and transcription factors[2].

ZEBRAFISH AS A MODEL ORGANISM

Zebrafish and humans experience very different environments except during their early development, where human embryos develop in an aquatic environment consisting of amniotic fluid[22]. All amniotic fluid derives from maternal blood, which transports nutrients, electrolytes, and water to the embryo and is a route of exposure to environmental factors including industrial

pollutants[23,24], and chemicals from household items[25,26] and lifestyle habits[27,28]. Exposure to environmental factors via amniotic fluid begins as early as two weeks post-conception and continues throughout gestation[29]. Therefore, performing waterborne exposures in the zebrafish embryo model replicates this environment and provides several advantages that have been described in a detailed review by Bugel et al. [22]. These advantages include the capacity to spawn hundreds of developmentally synchronized embryos in a single spawning event allowing simultaneous exposures in relatively small volumes, and a short duration of exposure, from one hour to a few days, which encompass multiple developmental processes due to the rapid growth rate of zebrafish embryos relative to humans (from fertilized egg to free-swimming hatchling in 3–5 days). Furthermore, zebrafish are genetically very similar to humans as approximately 70 % of human protein-coding genes have orthologs in the zebrafish, and over 80 % of human disease-associated genes have a zebrafish counterpart[30]. Additionally, zebrafish are an excellent model for neurobiology studies, especially during the initial phases of nervous system development[31]. To this end, chemically induced behavioral abnormalities at sublethal concentrations can be measured to elucidate the molecular and cellular bases of neurophysiological effects[17].

ADIPOGENESIS AND OSTEOGENESIS

The mesenchyme is a type of animal tissue consisting of loosely associated stellate-shaped cells, which in the trunk and posterior regions of the head derive from mesoderm, and in the face, jaws, and neck, originate from the neural crest.

Neural crest- and mesoderm-derived mesenchyme populations have many derivatives in common, but they also give rise to distinct cell and tissue types. Both make cartilage, bone, tendon, perivascular smooth muscle, glandular stroma, meninges, adipose tissue, and dermis.

Neural crest mesenchyme alone generates peripheral autonomic neurons, sensory neurons, peripheral glia, Schwann cells, calcitonin-producing C cells, melanocytes, odontoblasts, and cementoblasts. Mesodermal mesenchyme uniquely produces skeletal muscle, cardiac muscle, visceral smooth muscle, endothelium, endocardium, and serosa. The progression of neural crest and mesodermal mesenchyme into their terminal cell types involves similar developmental mechanisms.

Mesenchymal cells transition from multipotent to committed, but undifferentiated, blast cells before progressing onto their terminally differentiated cell types, including chondrocytes (cartilage), osteocytes (bone), and adipocytes (fat). Cells in each lineage are distinguished by the acquisition of unique phenotypes and protein expression profiles that enable the cells to perform their specialized functions. As the cells differentiate along these lineages, their proliferation decreases, and the fully differentiated cells stop dividing. The differentiation along each of these lineages is driven by the expression and activities of defined transcription factors that are regulated by signaling inputs and through interactions with other proteins. For example, Runx2, a runt domain transcription factor, functions as a key driver in osteogenic differentiation. Following Runx2 expression, there is upregulation of Osterix (osx) in the intermediate state of osteogenesis that results in a preosteoblast. As the cell moves from a preosteoblast to an immature osteoblast there is increased expression of secreted protein acidic and rich in cysteine (SPARC), secreted phosphoprotein 1 (SPP1, also known as OPN) and collagen type X alpha 1 chain (COL10A1).

Adipogenic differentiation requires the C/EBPs (CCAAT/enhancer-binding proteins) and PPARγ (peroxisome proliferator-activated receptor γ) proteins. C/EBPβ and δ, members of a larger family of transcription factors characterized by a basic leucine zipper (bZIP) domain,

activate the expression of PPARγ in preadipocytes. PPARγ is a nuclear receptor that functions through heterodimerization with another nuclear receptor, RXR (retinoid X receptor). PPARγ cooperates with C/EBPα to drive the expression of proteins that characterize the differentiated adipocyte.

INNER EAR DEVELOPMENT

The inner ear serves as a sensor, detecting head movements about an axis (horizontal, vertical, or torsional) and houses three semicircular canals, which respond to rotational acceleration (head turning), and two gravity receptor organs, which sense linear acceleration and gravity. The utricle and saccule are two gravity receptor organs that contain otoconia, consisting of dozens of tiny proteinaceous calcium carbonate ($CaCO_3$) bio-crystals. Otoconia are embedded in a membranous structure, the otoconial membrane, which lies above the kinocilia and stereocilia of hair cells in the sensory epithelium (macula) of the inner ear. In teleost fish, the bio-crystals are solidified into three otoliths; each directly interacts with an entire patch of sensory epithelium. Changes in the position of the head or linear acceleration result in displacement of the otoconial complex, producing a shearing force that deflects the attached kinocilia. At the base of the kinocilia are stereocilia that are attached via tip link proteins that respond to deflections of the kinocilia by opening mechanically gated calcium channels. The opening of these channels results in the subsequent depolarization of the sensory hair cells. Post-depolarization, electrical signals then travel to the central nervous system (CNS) by the afferent vestibular nerve, which integrates other proprioceptive information, to stimulate the CNS to initiate neuronal responses for maintaining body balance. The correct formation and anchoring of otoconia and otoliths are essential for optimal vestibular function and balance[32–36].

Otoconia-related balance disorders such as vertigo are prevalent, and dislocation of otoconia from the utricle to the semicircular canals is the most common cause in humans[37–39]. Otoconial abnormalities can have multiple causes such as genetic mutations, head trauma, and ototoxic xenobiotics or chemicals.

While zebrafish otoliths are structurally distinct from mammalian otoconia, genetic studies have shown that the zebrafish is an excellent predictive model for human vestibular development and disease[30]. Otoconia and otoliths contain $CaCO_3$; however, morphology, crystalline structure, and protein composition differ between the two types of biominerals. The otoconia have a barrel-shaped body with triplanar surfaces, each surrounded by an organic matrix[40–44]. In contrast, the dome-shaped fish otoliths contain alternating concentric layers of organic matrix and $CaCO_3$ crystal lattices[45]. The crystal lattice structure also differs: from advanced fish to higher vertebrates, the crystalline structure has evolved from aragonite to calcite, respectively[46,47]. Mammalian otoconia form during the late embryonic stages and may require some maintenance afterward[48,49]. After initial seeding, fish otoliths quickly attach to immature hair cells known as tether cells and rapidly grow during early ear development[50,51]. Daily growth layers, of varying thickness and composition, depending on the environment, are subsequently added to the otolith throughout the life of the fish[52]. Recent data support a model in which the $CaCO_3$ crystalline structure and morphology of the otoconia and otoliths are regulated by an organic matrix made of proteins and proteoglycans[53–55] (Fig. 1).

Otoconial proteins, collectively referred to as otoconins, many of which are essential for the $CaCO_3$ crystallization, bind calcium from the surrounding, calcium-poor endolymph. Nine murine otoconins have been identified: Otoconin-90, Otolin-1, Keratin sulfate proteoglycan(s), α-Tectorin, Fetuin-A, Osteopontin/ secreted phosphoprotein 1, Sparc-like protein 1, Osteonectin/

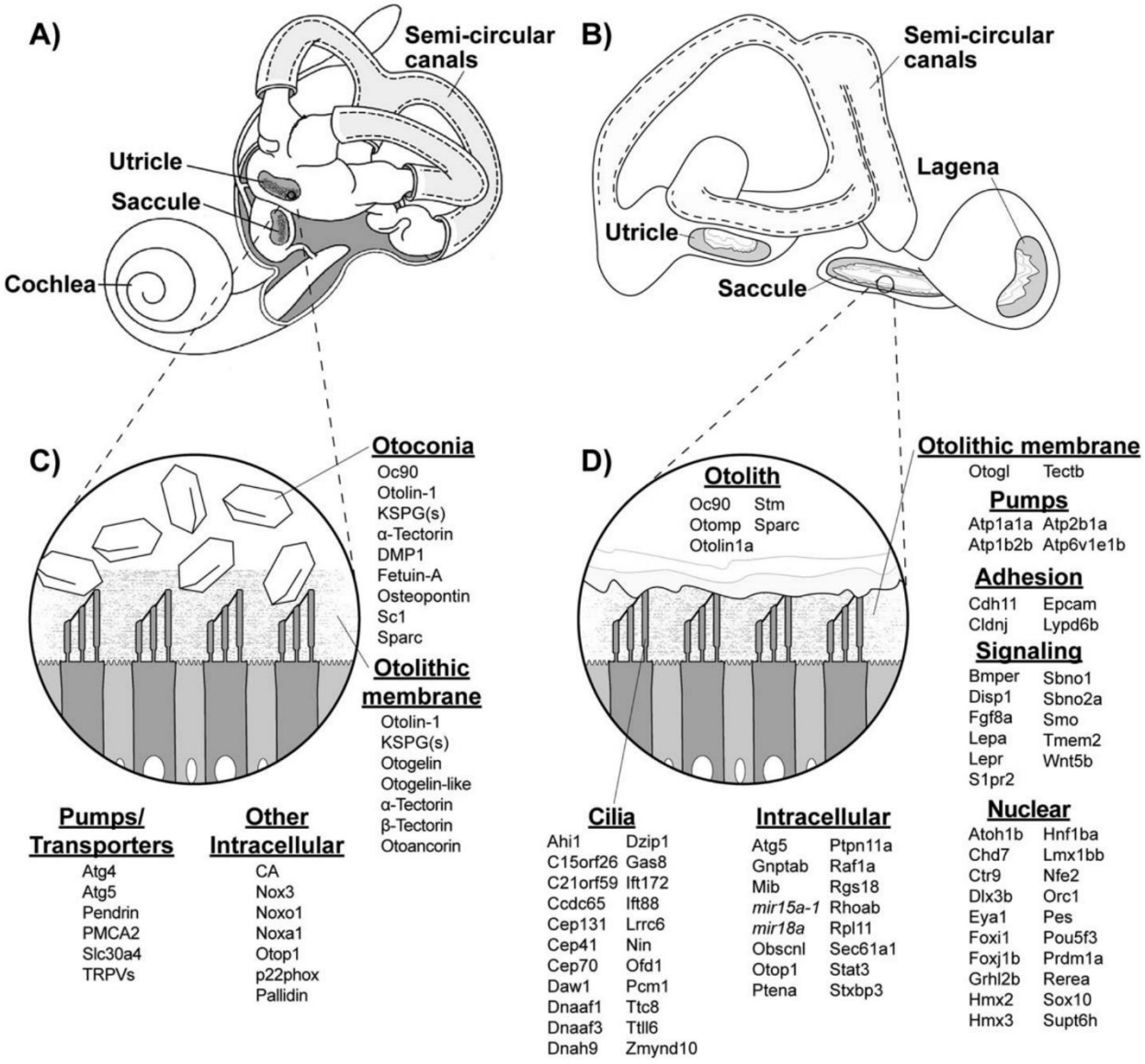

Figure 1: An illustration of the mammalian and zebrafish inner ear. Reproduced from Lundberg et al. (2015). (A) Mammalian inner ear. (B) Zebrafish inner ear. (C) Zoomed-in illustration of Otoconia in the mammalian vestibular space. (D) Zoomed-in illustration of Otoliths is the zebrafish vestibular space.

secreted protein acidic and cysteine rich, and Dentin matrix protein 1. Bio-crystal development in the inner ear is regulated by proteins that are not incorporated into otoconia and otoliths. These regulatory proteins are critical in establishing the appropriate environment for crystal seeding and growth by regulating otoconin secretion or post-translational modifications, as well as spatial and temporal control of calcium and other ions. The membrane-bound NADPH

oxidase (NOX) enzymes produce reactive oxygen species that have both normal and pathological roles. Plasma membrane calcium-ATPases (PMCAs) are calmodulin-dependent, and a vital source of calcium for otoconia formation as these pumps are Ca^{2+}/H^{+} exchangers that release Ca^{2+} from hair cell stereocilia, thereby increasing the Ca^{2+} concentration of the endolymph. Otopetrin 1 (OTOP1) is required for both otoconia and otolith development possibly by regulating protein secretion and mobilizing calcium[56,57]. In zebrafish, *otop1* is expressed in hair and supporting cells before otolith seeding, but only in hair cells during otolith growth[57,58]. Carbonic anhydrase (CA) likely regulates otoconia development and maintenance by providing HCO_3^- and maintaining an appropriate pH. Alteration of macular carbonic anhydrase is associated with changes in zebrafish otolith growth. A family of transient receptor potential vanilloids (TRPVs) selectively transport calcium and magnesium and may mediate endolymph homeostasis in the inner ear. Zebrafish *trpv4* is found in vestibular and cochlear sensory epithelia and the endolymphatic sac, whereas *trpv5* and *trpv6* are found in vestibular semi-circular canal ducts. The low endolymphatic pH in pendrin-deficient mice appears to inhibit acid-sensitive TRPV5/6 calcium channels, increasing endolymphatic calcium that likely contributes to the formation of abnormal otoconia crystals. Purinergic receptor P2X ligand-gated ion channels (P2RXs) are a family of nonselective ligand-gated purinergic receptor cation channels present in multiple species, from unicellular organisms to humans[59]. There are seven mammalian and zebrafish purinergic receptor subunits, denoted P2RX1 - P2RX7 and P2rx1 - P2rx8, respectively. There is no ortholog for P2RX6 in zebrafish or P2rx8 in mammals[59]. P2X receptors are expressed in many tissues and cells, where they are essential in mediating a diverse array of physiological processes from neurotransmission, muscle contraction and hearing to immune responses. P2RX2 is the only P2X receptor found to be expressed in the cochlea of the inner ear,

particularly in spiral ganglion neurons, epithelial cells in Reissner's membrane, and hair cell stereocilia in the organ of Corti[60–62]. These proteins and the otoconial regulators and ion channels are all highly conserved throughout evolution increasing the utility of the zebrafish as a model for ototoxicity.

NEURODEVELOPMENT

Neurodevelopmental disorders (NDDs) are defects in growth and or development of the central nervous system; they can be caused by genetic or environmental factors. The latter can include physical trauma, xenobiotic exposure, and biological causes such as viral or bacterial infections during critical periods of nervous system development. In humans, manifestations of neurodevelopmental disorders are extensive and complex and include intellectual disabilities, communication disorders, traumatic brain injuries, autism spectrum disorders, epilepsies, and motor and coordination disorders. Many of these human disorders appear to have model organism counterparts in rodents and fish, enabling experiments designed to elucidate the mechanistic bases of their origins.

Neurotoxicity occurs when exposure to toxic substances alters the regular activity of the nervous system including effects on the central and peripheral nervous system or sensory organs.[63] Testing for neurotoxicity and developmental neurotoxicity in animal models is becoming more and more relevant as exposure to environmental contaminants increases, as well as the increasing number of people with neurological disorders[64]. It has been shown that neurological diseases like Parkinson's disease or autism spectrum disorder can be induced by exposure to environmental chemicals[65,66]. So far, the neurotoxic potency of chemicals has mainly been determined with neurobehavioral and neuropathological *in vivo* tests in rodents. These tests

are time-consuming and expensive. Several *in vitro* tests are available but are mostly a complement to *in vivo* studies as they cannot deal with toxicokinetic aspects such as uptake and metabolism. *In vitro* tests are generally used to identify mechanisms of toxicity of suspected neurotoxicants.

Unlike other organs that are formed at a specific developmental stage, the nervous system develops throughout all stages and even during adulthood[64,67]. Therefore, the effects of neurotoxic substances can produce very different phenotypes depending on the timing of exposure, and the developing nervous system is more vulnerable to chemicals than the adult nervous system[67]. This vulnerability stems from the fact that the nervous system must develop from a strip of cells along the dorsal ectoderm into a complex organ consisting of billions of precisely located, highly interconnected, and specialized cells without the protection of a fully formed blood brain barrier[67,68]. Therefore, impacts of neurotoxicants on neurogenesis, cell proliferation, cell migration or natural apoptotic processes can lead to harmful effects on the morphology of the developing nervous system[63]. This can result in neurodevelopmental disorders causing lifelong disability, stressing the importance of identifying and restricting exposure to compounds with developmental neurotoxic potential[63].

One of the oldest and most studied developmental neurotoxicants is lead (Pb)[64]. Pb is known to interfere with the regulatory action of calcium in cell functions resulting in apoptotic cell death, impaired neuronal differentiation, and the trimming/pruning of synaptic connections [64,69,70]. At similar concentrations developmental Cd exposure results in neuronal apoptosis, impaired neurogenesis, and altered neuronal gene expression and epigenetic effects[17]. Like Pb, Cd can alter the intracellular concentration of calcium but many reports indicate that the toxic mechanisms of cadmium act through the accumulation of reactive oxygen species (ROS)[68,71].

However, the exact mechanism(s) through which cadmium causes its neurotoxic effects is still unresolved.

RESEARCH OBJECTIVES AND APPROACH

The approaches outlined below focus on how developmental cadmium exposure alters behavior and cell fate by disruption of the vestibular system and mesenchymal stem cell differentiation, respectively. The hypothesis underlying each approach is that developmental cadmium exposure at non-occupational levels, is capable of affecting multiple developing biological systems resulting in altered adipogenic and osteogenic development, and aberrant behavioral responses. Based on the data presented in chapters 2, 3, and 4, we expand upon the current literature demonstrating that cadmium is a developmental obesogen and ototoxicant in two alternative toxicological models: zebrafish (*Danio rerio*), a teleost model of human adipose, bone and vestibular development; and human adipose-derived mesenchymal stem cells, a multipotent stem cell model capable of adipogenic, chondrogenic and osteogenic differentiation *in vitro*.

Cadmium exposure increases the risk of juvenile obesity: a human and zebrafish comparative study

There was modest evidence showing that cadmium exposure in adult animals was associated with metabolic syndrome both *in vitro* and *in vivo*, and antiobesogenic activity by promoting the release of lipids from hepatic and adipose tissue, resulting in dyslipidemia. Whereas prenatal exposure may increase the risk of lipid accumulation, there are limited data evaluating the association between developmental exposure to cadmium and obesity. Therefore,

the aim of chapter 2 was to investigate the effects of developmental cadmium exposure on body weight and abdominal lipid accumulation in children and juvenile zebrafish, respectively. To test this hypothesis cadmium was measured in first-trimester blood samples from infant-mother pairs and logistic regression was implemented to evaluate the association between childhood obesity and the concentration of cadmium. As episodic growth acceleration can induce bias, we adjusted for child weight trajectory from birth to 36 months using growth trajectories computed as growth curves for each child, and functional principal component analysis (FPCA) was implemented to summarize growth curves. In addition, AB wild-type zebrafish embryos were exposed to cadmium from four hpf through seven dpf and evaluated for lipid accumulation at one and two months post-fertilization using Nile red staining.

Cadmium interferes with mesenchymal stem cell development

To understand the mechanisms behind the changes in lipid accumulation observed in chapter 2 along with cadmium's known role in bone toxicity in adults, chapter 3 aims to show that cadmium exposure disrupts osteogenic or adipogenic differentiation of mesenchymal stem cells resulting in delayed bone formation and increased lipid accumulation *in vitro* and *in vivo*.

To address these goals, human adipose-derived mesenchymal stem cells were exposed to cadmium in the presence of adipogenic differentiation media for four days followed by adipose maintenance media with cadmium for 17 to 19 days (until lipid droplets were apparent). Assessment of adipogenesis was performed by Oil Red O staining and imaging, and absorbance quantification assays of isopropanol-extracted Oil Red O were conducted at the end of the exposure. To assess changes in bone formation *in vivo,* AB wild-type embryos were exposed to cadmium from four hpf through seven dpf and evaluated for early, intermediate, and apical

stages of differentiation, including: i) targeted gene expression of osteogenic regulators during exposure and, ii) Alizarin Red staining for mineralized bone at 8 and 14 dpf.

Developmental cadmium exposure disrupts vestibular calcium channels interfering with otolith formation and inner ear function.

In chapter 4 we investigated whether cadmium induces changes in the vestibular system and how these changes result in aberrant behavioral responses. The overall aim of this chapter was to show that developmental cadmium exposure induces vestibular disruption resulting in behavioral alterations and increased sensitivity to stimuli. To test this hypothesis, AB wild-type embryos were exposed to cadmium from four hpf through five dpf, and abnormal rotational behavior in response to dark-light cycles was measured manually in the larval zebrafish. Assessment of developmental cadmium exposure and the role it plays in disrupting normal vestibular function was performed using startle response experiments on five dpf larvae followed by analysis of the startle frequency. Cadmium is known to interact with calcium in other organ systems in zebrafish and other animal models. Therefore, we used inner ear calcium channel agonists and antagonists to determine the mechanism by which cadmium disrupts otolith formation in the vestibular system. Zebrafish embryos were exposed to cadmium with or without P2rx or Trpv6 agonists and antagonists from four hpf through seven dpf, at which time the size of their otoliths was assessed for cadmium-induced changes.

Developmental cadmium exposure induces ROS dependent hyperactivity in larval zebrafish

One mechanism of cadmium toxicity identified in the literature is the production of ROS. Cadmium is unable to generate free radicals directly but instead facilitates the production of ROS through depletion of the ROS scavenger molecule glutathione, mitochondrial damage, induction of NADPH oxidases, and replacement of iron, a redox-active element[18]. ROS have been shown to negatively impact neurodevelopment resulting in cognitive impairment in animal models. Therefore, the aim of appendix A was to test whether cadmium is capable of inducing behavioral changes in larval zebrafish in a concentration-dependent, ROS-mediated manner. To test this hypothesis, AB wild-type embryos were exposed to cadmium with or without antioxidants from four hpf through five dpf and activity in response to dark-light cycles was measured. In addition, targeted genetic markers of oxidative stress were assessed.

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CHAPTER 2

Cadmium Exposure Increases the Risk of Juvenile Obesity: A Human and Zebrafish Comparative Study

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Abstract

OBJECTIVE: Human obesity is a complex metabolic disorder disproportionately affecting people of lower socioeconomic strata, and ethnic minorities, especially African Americans and Hispanics. Although genetic predisposition and a positive energy balance are implicated in obesity, these factors alone do not account for the excess prevalence of obesity in lower socioeconomic populations. Therefore, environmental factors, including exposure to pesticides, heavy metals, and other contaminants, are agents widely suspected to have obesogenic activity, and they also are spatially correlated with lower socioeconomic status. Our study investigates the causal relationship between exposure to the heavy metal, cadmium (Cd), and obesity in a cohort of children and in a zebrafish model of adipogenesis.

DESIGN: An extensive collection of first trimester maternal blood samples obtained as part of the Newborn Epigenetics Study (NEST) was analyzed for the presence of Cd, and these results were cross analyzed with the weight-gain trajectory of the children through age five years. Next, the role of Cd as a potential obesogen was analyzed in an *in vivo* zebrafish model.

RESULTS: Our analysis indicates that the presence of Cd in maternal blood during pregnancy is associated with increased risk of juvenile obesity in the offspring, independent of other variables, including lead (Pb) and smoking status. Our results are recapitulated in a zebrafish model, in which exposure to Cd at levels approximating those observed in the NEST study is associated with increased adiposity.

CONCLUSION: Our findings identify Cd as a potential human obesogen. Moreover, these observations are recapitulated in a zebrafish model, suggesting that the underlying mechanisms may be evolutionarily conserved, and that zebrafish may be a valuable model for uncovering pathways leading to Cd-mediated obesity in human populations.

Introduction

The prevalence of obesity has more than doubled among children and more than tripled among adolescents in the last 30 years[1,2]. While obesity prevalence has plateaued overall in the last two years, the disparities in the prevalence of obesity in children of lower socioeconomic status (SES) and racial/ethnic minorities appear to be widening[3-5]. Genetic predisposition and energy imbalance, where caloric input exceeds energy output, are implicated in obesity; however, these factors alone cannot explain the disproportionate incidence of obesity in lower SES populations. The increased use of organic and inorganic chemicals for a wide range of applications in the last century has been paralleled by increases in the body burden of environmental pollutants, many of them endocrine disruptors. In animal models, *in vitro* and in humans, many of these chemicals have been associated with lipid accumulation and progressive cardiometabolic dysfunction. However, these data have been difficult to interpret and use to recommend public action, as the specificity of the associations between many of these chemicals and the cardiometabolic disease risk phenotype has not been demonstrated, and the doses of exposure in model systems are often at or above human occupational levels.

Cadmium (Cd) is a ubiquitous environmental contaminant ranked seventh on the list of toxicants of concern by the Agency for Toxic Substances and Disease Registry (ATSDR)[6]. Two to three decades leading up to the 1970s saw a rapid increase in the use of Cd in the manufacture of fertilizer and nickel-cadmium batteries, that paralleled an increase in blood Cd concentrations in the US population[7-10]. Major sources of human exposure include ingestion of foods contaminated with Cd, cigarette smoke, and breathing contaminated air in occupational settings or in neighborhoods near contaminated industrial facilities. The mechanisms by which Cd elicits toxicity are not entirely clear, although induction of oxidative stress has been implicated.

Understanding the connection between exposure and Cd-mediated outcomes may be further complicated by its long half-life, estimated to be between 10 and 45 years, in the kidney, liver, lung and pancreas[11,12]. Cd is a known human carcinogen and is associated with respiratory, renal, neurological, and bone disorders. In addition, some studies[13-15], including reviews[12,16-18], but not others[19,20] link lower levels of Cd to cardiovascular and metabolic diseases; however, these associations are limited to adults.

Epidemiological and animal studies over the past 15 years have demonstrated that *in utero* and neonatal environmental exposures alter programming of endocrine systems involved in growth, energy metabolism, adipogenesis, appetite, and glucose-insulin homeostasis of the developing fetus[21-25]. Cd exposure has been associated with lower birth weight[26-28], a phenomenon known to be a persistent risk factor for accelerated adiposity gain in young children, which has been linked to cardio-metabolic impairment in adulthood[29-35]. Exposures occurring during critical developmental windows have been shown to stably alter the function of target organ systems, and initiate processes that increase the risk of cardiometabolic diseases later in life[29,36]. Currently cohort data linking low-level prenatal Cd exposure to cardiometabolic outcomes are limited and derive from studies with short follow-up[37-39]. Thus, it remains unclear whether early indications of metabolic dysfunction that have been associated with developmental exposure to Cd persist into middle childhood or adulthood. Furthermore, because prenatal Cd exposure also disproportionately affects lower SES strata, disentangling the contributions of Cd from competing risk factors including physical activity, dietary patterns, and other non-chemical stressors, has thus far not been possible[40]. Additional models are needed to isolate the effects of early developmental exposure to Cd on metabolic indicators.

Zebrafish (*Danio rerio*) is a powerful model system for toxicological research[41,42]. Its genome is sequenced and its conservation with humans is facilitating mechanism-based understanding of chemical effects on diverse human conditions[43]. Its experimental strengths include its small size, high fecundity, availability of transgenic lines for live imaging of complex physiological processes, embryonic transparency, experimental tractability, and conserved but simplified anatomy[41,42]. Zebrafish larvae and adults are semitransparent and offer unique opportunities to study the effects of environmental exposures on adipogenesis and metabolic function *in vivo*[44]. Adipose tissue is recognized as a dynamic endocrine organ that plays a critical role in regulating metabolic homeostasis[45], in addition to storing excess fat. Adipose tissue is first detected in zebrafish at about two weeks post-fertilization, embryonic and early larval stages are sensitive to compounds that modulate fat metabolism[44, 46-48]. The deposition and mobilization of lipid within zebrafish adipose tissue can be altered by nutritional manipulation, suggesting that energy storage functions of adipose tissue are conserved between zebrafish and mammals[49]. In addition, gene expression studies on unfractionated zebrafish adipose tissue show shared pathophysiologic pathways indicating that zebrafish studies involving adipogenesis and metabolic function may be directly translatable to humans[49, 50].

Here, we present human data linking prenatal Cd exposure to obesity in children at five years of age, and demonstrate that this effect is recapitulated in juvenile zebrafish exposed to Cd during the larval stage. Despite the likely presence of confounders in the human data, our findings in zebrafish, in which the exposure profile is strictly controlled, demonstrate for the first time that Cd may be a human obesogen, and that prenatal human exposure to Cd likely initiates a cascade of molecular events leading to increased adiposity.

Materials and Methods

Study participants

Study participants were pregnant women enrolled in the Newborn Epigenetic STudy (NEST), a prospective cohort study of women and their offspring enrolled from 2009 to 2011 from six prenatal clinics in Durham County, North Carolina. Participant accrual procedures were previously described[51, 52]. Briefly, inclusion criteria were: age 18 years or older, pregnant, and intention to use one of two participating obstetric facilities in Durham County for delivery. Exclusions were: plans to relinquish custody of the index child, move states in the subsequent three years, or an established HIV infection. In the 18-months beginning April, 2009, 2,548 women were approached and 1,700 consented (66.7% response rate). The present analyses are limited to the first 319 infant-mother pairs in whom we measured first trimester blood Cd, arsenic (As) and lead (Pb). Maternal race, smoking status, BMI before pregnancy, parity, delivery route, and education were comparable in the 319 infant-mother pairs included in this study and the remainder of the cohort ($p>0.05$). The study protocol was approved by the Duke University Institutional Review Board.

Data and specimen collection

Participants completed a self- or interviewer-administered questionnaire at the time of enrollment that included social and demographic characteristics, reproductive history, lifestyle factors, and anthropometric measurements. At study enrollment, maternal peripheral blood samples were collected; the mean gestational age at maternal blood draw was 12 weeks. Blood aliquots were prepared and stored at −80°C.

Measurement of cadmium

Prenatal Cd blood levels were measured in whole blood as nanograms per gram (ng/g; 1000ng/g=1035ng/µl) using well-established solution-based ICP-MS methods[53-56]. Procedures were described previously[26]. Briefly, frozen maternal blood samples were equilibrated at room temperature, homogenized with a laboratory slow shaker (GlobalSpec, East Greenbrush, NY) and ~0.2 mL aliquots were pipetted into a trace-metal-clean test tube and verified gravimetrically to ±0.001mg using a calibrated mass balance. Samples were spiked with internal standards consisting of known quantities (10 and 1 ng/g, respectively) of indium (In) and bismuth (Bi) (SCP Science, USA), used to correct for instrument drift. The solutions were then diluted using water purified to 18.2 MΩ/cm resistance, hereinafter referred to as Milli-Q water (Millipore, Bedford, Mass., USA) and acidified using ultra-pure 12.4 mol/L hydrochloric acid to result in a final concentration of 2% hydrochloric acid (by volume). All standards, including aliquots of the certified NIST 955c, and procedural blanks were prepared by the same process.

Cd concentrations were measured using a Perkin Elmer DRC II (Dynamic Reaction Cell) axial field ICP-MS at the University of Massachusetts-Boston[53-56]. To clean sample lines and reduce memory effects, sample lines were sequentially washed using Milli-Q water for 90 seconds and a 2% nitric acid solution for 120 seconds between analyses. Procedural blanks were analyzed within each block of 10 samples, to monitor and correct for instrument and procedural backgrounds. Calibration standards used to determine metal in blood included aliquots of Milli-Q water, and NIST 955c SRM spiked with known quantities of each metal in a linear range from 0.025 to 10 ng/g. Standards were prepared from 1000 mg/L single element standards (SCP Science, USA). Method detection limits (MDLs) were calculated according to the two-step approach using the $t_{99}S_{LLMV}$ method (USEPA, 1993) at 99% CI (t=3.71). The MDLs yielded

values of 0.006, 0.005, and 0.071 µg/dL, for Cd, Pb, and As, respectively. Limits of detection (LOD) were 0.002, 0.002, and 0.022 µg/dL, for Cd, Pb and As, respectively, and limits of quantification (LOQ) (according to Long and Winefordner, 1983) were 0.0007, 0.0006, and 0.0073 µg/dL for Cd, Pb, and As, respectively. The number of samples below the LOD for Cd, Pb, and As were two, two, and one, respectively.

Statistical analyses

Childhood obesity at age five was defined by the weight-for-height z score (WHZ)[57]. Children with WHZ scores greater than 85% of their same sex peers at age five were classified as overweight/obese. Logistic regression was implemented to evaluate the association between childhood obesity and the concentration of Cd, adjusting for other co-occurring metals (Pb and As) in maternal blood, maternal smoking (never, quit during pregnancy, pregnant smoker), breastfeeding (over three months or less), and sex of child. To reduce bias related to episodic growth acceleration, we additionally adjusted for child weight trajectory from birth to 36 months. These growth trajectories were computed as growth curves for each child, and functional principal component analysis (FPCA) was implemented to summarize growth curves. In the final model the top three FPCs, which explain >95% of the variability in the original growth curves, were included as covariates in the regression model; modeling with the top two FPCs did not alter the conclusions and produced a modest decrease in accounting for variability (94%). Similar to PCA (which aims to extract orthogonal PCs that retain maximal amount of variation in the original variables by estimating the eigenvalues and eigenvectors of the sample variance-covariance matrix), FPCA aims to obtain orthogonal functional PCs that retain the maximal

amount of variation in the original weight curves by estimating the eigenvalues and eigenfunctions of the sample variance-covariance function.

Zebrafish husbandry and embryo collection

Wildtype (AB) zebrafish were maintained in a zebrafish facility at NC State University according to standard protocols,[58] and in conformity with guidelines of the NC State Animal Care and Use Committee (ACUC), which also approved all animal experiments reported. Briefly, adults were maintained at 28.5° C and a 14/10-hour light/dark cycle, and fed a standard diet twice daily. Spawning took place at a ratio of three females to one male; embryos were collected every 30 minutes and scored for viability prior to use in downstream applications.

Radioassay to assess cadmium uptake by larval zebrafish

To assess total body concentrations of Cd in zebrafish, triplicate groups of zebrafish embryos (n=25/group) were exposed from four hours post-fertilization (hpf) to seven days post-fertilization (dpf) to 60 μg/L (60 ppb) Cd in the form of $CdCl_2$ (Cd mass fraction: 0.613; 0.534 □M $CdCl_2$) in 0.5x embryo media (E2), spiked with ^{109}Cd as a tracer (1592 Bq μg^{-1}). Solutions were replaced daily during the course of the experiment. Larval uptake of Cd was monitored daily beginning at three dpf by measuring radioactive decay corrected for background activity. Briefly, larvae were washed three times with five ml of Cd-free, non-radioactive 0.5x E2 media followed by transfer to clean scintillation vials in two mL of the final wash. An additional two mL of the final wash were transferred to a second clean scintillation vial to measure background activity. The radioactivity uptake was measured using a Wallac Wizard 1480 Gamma counter. All larval measurements had counting errors <5%.

Cadmium and lead exposure

Cohort: Although the sources of Cd are not known with certainty, we previously reported detecting Cd in soil samples collected from neighborhoods of pregnant women with elevated Cd blood levels, but not in water. However, the correlation between Cd concentrations in soil and blood were weak and not significant (King, 2015) suggesting other unmeasured sources such as house dust or ambient air may also contribute as sources. *Zebrafish:* Stock solutions of $CdCl_2$ ([Cd], 99.99% purity; Sigma-Aldrich, MO) were made at 60 parts per million in Milli-Q water; stock solutions of Pb(II) acetate ($Pb_2(C_3O_2H)_2$; Sigma-Aldrich, MO) were made at 100 ppm in milliQ water. Zebrafish embryos were collected as described and exposed to 60 ppb Cd or 100 ppb Pb in 0.5X embryo media[58] from four hpf to seven dpf at a density of 10 embryos/mL with daily replacement, and fed beginning at five dpf. After removal of Cd or Pb, larvae were raised for lipid content analysis at one and two months post-fertilization.

Lipid analysis

The vital dye, Nile red, was used to stain lipids in juvenile zebrafish (one and two months post-fertilization), which allows repeated analysis of the same individual to assess amount and location of lipid droplets over time[49]. A 1.25 mg/mL stock solution was made in acetone. Immediately before use, a working solution was made by diluting 10 µL of the stock solution into 25 mL of aquarium system water to provide a final concentration of 0.5 µg/mL. Live zebrafish were stained in the dark for 30 minutes at 28˚C[44, 49]. Fish were removed from the Nile red solution and anesthetized in aquarium system water containing 0.25 mg/mL phosphate buffered (pH 7) Tricaine-S (Western Chemical, Ferndale, WA).

Imaging and quantitative analysis

Nile red-stained zebrafish were imaged using a Leica MZ FLIII fluorescence stereomicroscope. Images were analyzed using Fiji[59]. Color thresholding was used to select Nile red-containing sections by setting the hue value at 20-50. Background fluorescence was removed by setting a minimum brightness threshold of 120. Remaining fluorescence was selected and analyzed using the measure tool[44, 60, 61]. To account for differences in body size, fluorescence was normalized by taking the ratio of fluorescence to the dorsal-ventral height at the point where the anal fin attaches anteriorly to the body[62].

Results

Study subjects

The distributions of first trimester blood Cd concentrations were compared by social and demographic characteristics of the mother-child pairs (Table 1). The Cd geometric means were comparable to the U.S. population; however, our cohort included a geographically clustered group of women with blood Cd levels at or above reportable levels, and we have complied with the reporting requirements. African Americans comprised 35% of the study population while Whites, Hispanics and Others comprised 30%, 32% and 4%, respectively. Nearly two thirds were younger than 30 years; approximately half had at least a high school education level, and reported a household income of at least $25,000 per year. Seventy-three percent were married or living with a partner. Fifteen percent of mothers reported smoking during pregnancy and 55% were overweight, obese, or extremely obese (29%, 15%, or 11% respectively). The majority of offspring (89%) had a birth weight within normal range (2.5 to 4 kg) and 88% were born at term. Blood Cd and Pb concentrations did not vary by maternal age, obesity, gestational age at delivery, or by sex and birth weight of offspring. However, blood levels of these heavy metals were higher among infants born to African Americans, Asians and Hispanics compared to Whites ($p=0.03$), smokers ($p=0.01$), and those who were obese before pregnancy ($p=0.02$). These factors were considered as potential confounders.

Table 1. Description of characteristics for study participants

Category		N	Cadmium (ng/g) quantile: median [IQR*]	Lead (ng/g) quantile: median [IQR*]
Maternal age (in years)	<30	182	0.1 [0, 0.2]	1.6 [0, 3.5]
	30<35	76	0.1 [0, 0.2]	1.5 [0.5, 2.8]
	35+	56	0.1 [0, 0.1]	2.0 [0.4, 5]
Maternal educational levels	Less than high school or high school	162	0.1 [0, 0.3]	2.1 [0, 4.1]
	College	151	0.1 [0, 0.1]	1.4 [0.4, 2.9]
	Graduate degree	1	0.2 [0.2, 0.2]	1.7 [1.7, 1.7]
Ethnic composition	White	96	0.1 [0, 0.1]	1.3 [0.4, 2.4]
	Black	108	0.1 [0, 0.3]	1.6 [0, 3.3]
	Hispanic	98	0.1 [0, 0.2]	2.2 [0, 4.9]
	Other	12	0.1 [0, 0.2]	2.7 [0.7, 5.3]
Cigarette smoking	Never Smoked	228	0.1 [0, 0.2]	1.5 [0.4, 3.5]
	Smoking during pregnancy	46	0.3 [0, 0.4]	1.7 [0, 3.2]
	Smoking prior to pregnancy only	40	0.1 [0, 0.2]	1.7 [0, 2.6]

**IQR: interquartile range

Table 2. Adjusted regression coefficients for associations between cadmium exposure and obesity parameters, in children at age 4-5 years*.

Parameter	Regression Coefficient	Std. Error	p-value
Intercept	6.085	3.655	0.096
Functional principal components for growth trajectories**	1.323	2.846	0.004
Functional principal components for growth trajectories	1.396	1.50	0.353
Prenatal blood Cd concentrations	3.184	1.296	0.014
Prenatal blood As concentrations	-18.466	9.396	0.049
Prenatal blood Pb concentrations	-0.007	0.070	0.925

* Model also adjusted for cigarette smoking, sex, and breastfeeding for at least 3 months.

** Functional principal components summarize growth trajectories form birth to age 3 years and are mutually exclusive.

Associations between first trimester cadmium and obesity

Maternal first trimester blood Cd concentrations were 0.3 ng/g of blood weight (IQR0.1-0.7), i.e. 0.03µg/dL, which is comparable to the US population[63]. Higher prenatal Cd levels were associated with higher obesity risk at five years of age (Table 2). The effect of Cd (β=3.18, se=1.30, p=0.014) was robust and corresponds to a ~25-fold increase in obesity odds at age five for every one ng/g increase in blood weight of Cd. These analyses were adjusted for prenatal cigarette smoking, blood concentrations of As and Pb, offspring sex, and breastfeeding, and the first three functional principal components of growth trajectories. Figure 1 also shows the increase in the magnitude of the adjusted associations between first trimester Cd exposure and obesity at each month with increasing age, until 30 months when it plateaus, indicating that Cd-associated obesity is likely sustained, at least in childhood. Furthermore, this pattern of association persisted among non-smokers when these analyses were repeated in the absence of the 10 smokers (β=3.55, se=2.00, p=0.077), and was independent of the persistent and monotonic relationship between growth trajectories and obesity observed in Figure 1. Additional adjustment for pre-pregnancy obesity did not alter these associations.

Cadmium uptake by larval zebrafish

Larval zebrafish began to accumulate measurable amounts of Cd from three dpf onward (Figure 2). The delay in Cd uptake correlated with the presence of the chorion, an embryonic membrane surrounding the developing embryo that typically ruptures at or about 48 hpf. Beginning at three dpf, Cd accumulation was approximately linear, and at seven dpf the total body burden of Cd reached 0.54 ng ± 0.1 ng/larvae. On average, a seven dpf larval zebrafish weighs 1.4 mg (wet weight)[64]; by extrapolation, this equates to 386 ng Cd per gram of larvae.

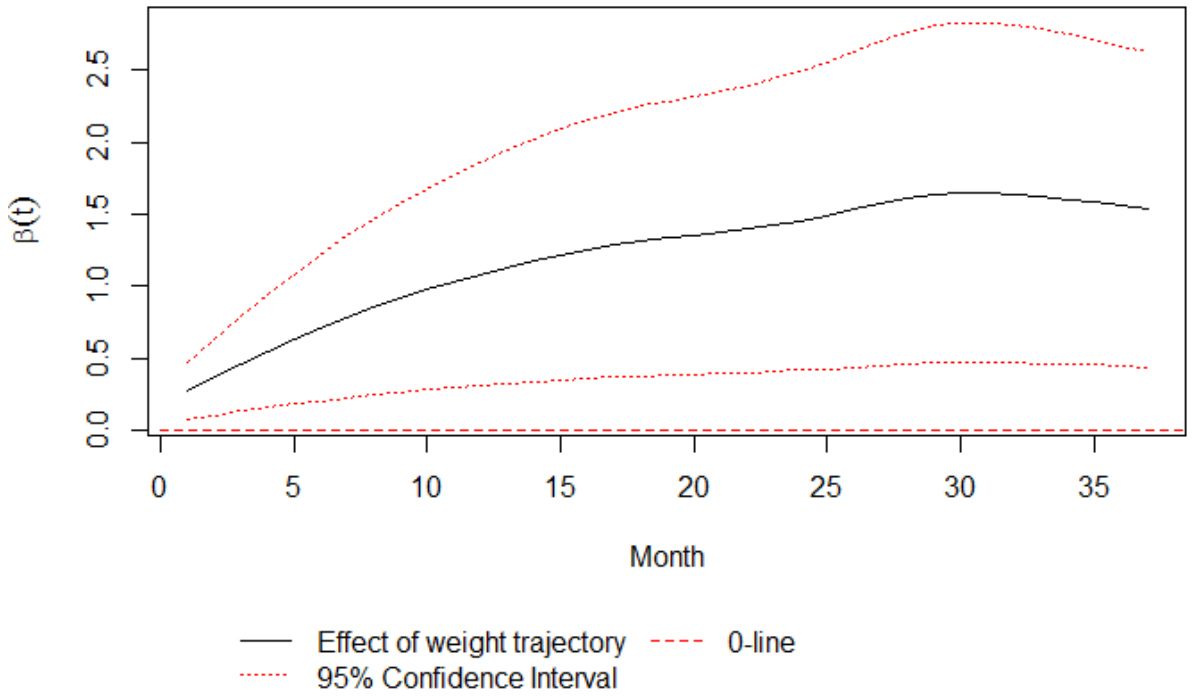

Figure 1. Effect of weight trajectory (via the first FPC) on obesity risk at age five. The solid line indicates the effect of child weight by month via the first FPC on obesity risk at age five; the flanking dashed lines represent the 95% simultaneous confidence band of the weight effect, accounting for multiple comparisons of all months; the dotted line indicates zero effects. The simultaneous confidence band lies above zero, indicating a significant, positive effect of child weight on obesity risk at age five. The solid line also suggests that the magnitude of the weight effect increases over time.

Since Cd burden is commonly reported as a serum concentration, we used the Cd toxicokinetic model proposed by Kjellström and Nordberg[65] to estimate a larval serum concentration. This model estimates that 0.06% of the total body burden of Cd can be found in the serum; therefore, the calculated serum concentration per larvae is 0.23 ng/g, in agreement with the values observed in the NEST cohort.

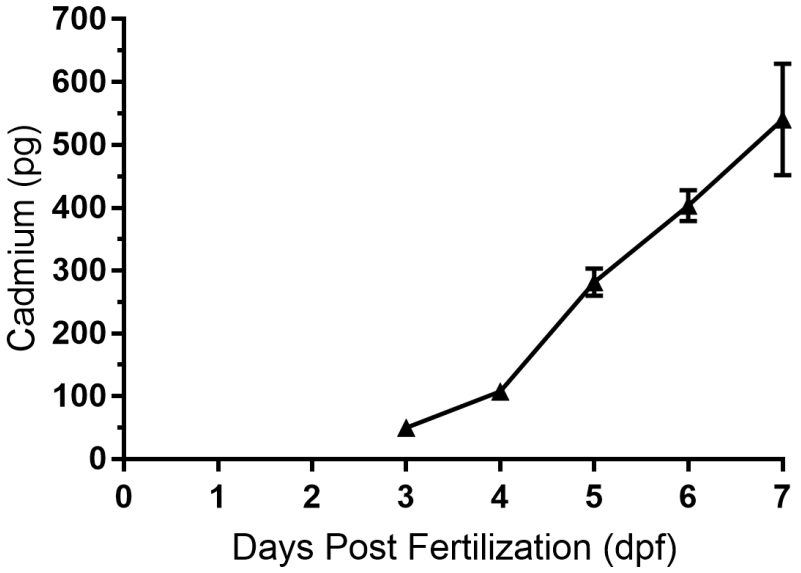

Figure 2. Total cadmium uptake during zebrafish development. Total internal Cd was measured as described after zebrafish embryos were exposed continuously from four hpf to seven dpf to Cd spiked with ^{109}Cd. Measurements began at three dpf after hatching from the chorion, which provides a significant barrier to Cd uptake. Measurements are mean ± SEM.

Cadmium-induced juvenile lipid accumulation

Zebrafish undergo rapid development, with free-feeding larvae emerging after five dpf. However, a prolonged juvenile period of approximately three months follows, resulting in sexually mature adults at about 3-3.5 months post-fertilization. Based on the Cd uptake assay, we exposed zebrafish to 60 ppb Cd during embryonic/larval development (0-7 dpf), at which point their estimated Cd plasma burden is in agreement with values measured in the NEST cohort. The exposed fish had significantly increased lipid accumulation at one and two months post-exposure as seen in size-adjusted Nile red fluorescence following exposure from four hpf to one week post-fertilization (Figure 3, $p < 0.05$). This increase in Nile red fluorescence was not seen at 3.5 months post-fertilization (data not shown) at which point the Nile red fluorescence was significantly decreased in the Cd-exposed group vs controls ($p < 0.01$). These data indicate that limited (developmental) exposure to Cd results in increased lipid accumulation in juvenile

zebrafish, which persists throughout the pre- and peri-pubertal stages but likely reverses at or before the onset of sexual maturity in the absence of continuous exposure. Unlike the results from Cd exposure, Pb at 100 ppb had no effect on adiposity (Fig. s1).

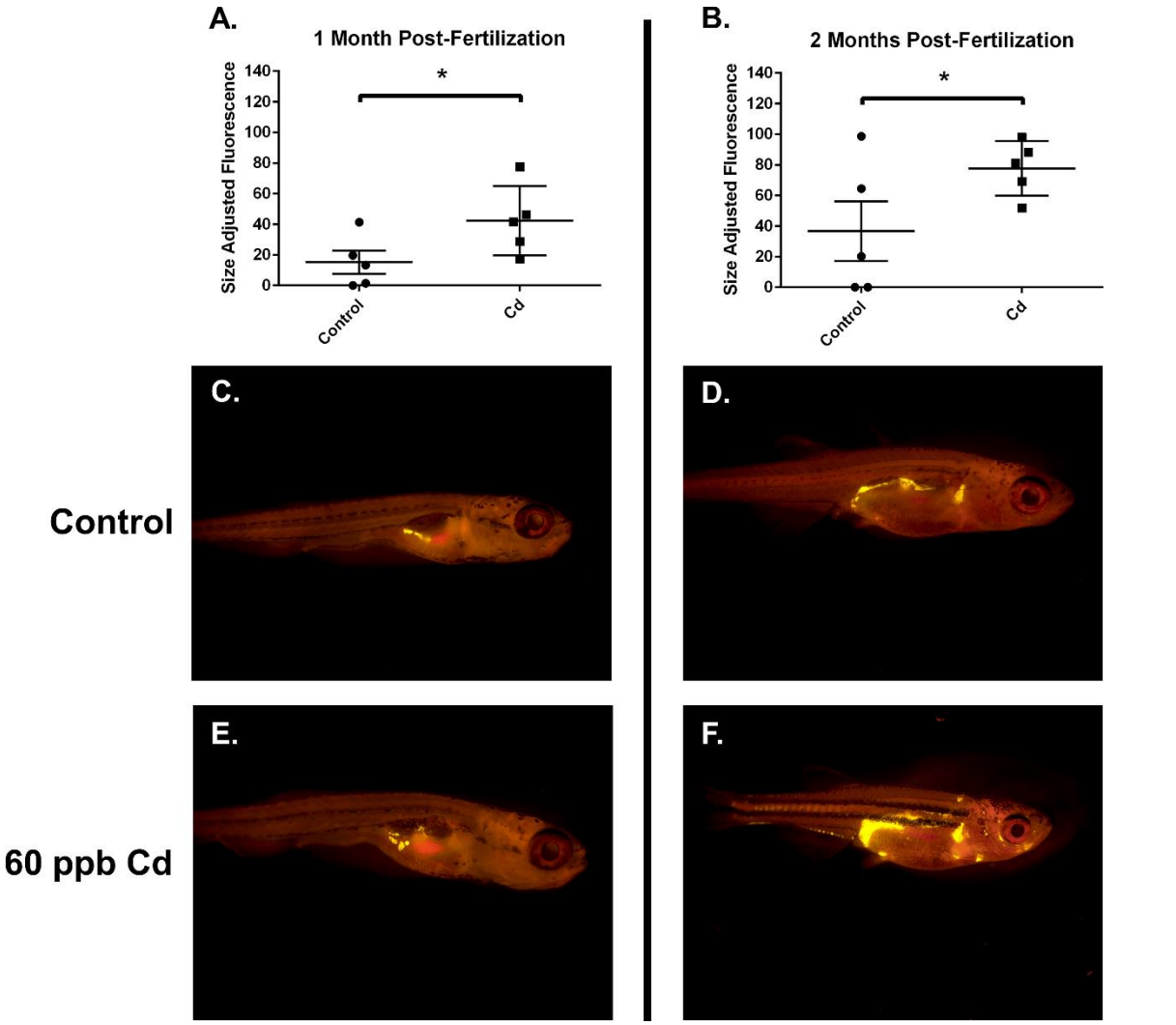

Figure 3. Developmental exposure to cadmium increases lipid deposition in juvenile zebrafish. Nile red fluorescence was significantly greater in zebrafish larvae exposed to 60 ppb Cd vs. water controls at one (A) and two (B) months post-fertilization ($p<0.05$). Representative live images of Nile red staining are shown for control (C, D) and Cd-exposed (E, F) zebrafish at one- and two-months post-fertilization, respectively.

Discussion

Although genetic predisposition and energy imbalance, where energy input exceeds output, are established risk factors fueling the obesity epidemic in children, caloric excess and physical inactivity alone fail to fully account for the magnitude and the steep trajectory followed by the obesity epidemic[66]. A growing consensus suggests that exposure to some lipophilic or metalloid contaminants is obesogenic; the most studied are persistent organic compounds such as polychlorinated bisphenyls[67], and metalloids such as arsenic[68-71]. However, the obesogenic potential of ubiquitous inorganic metals, including Cd, is unclear.

We evaluated associations between prenatal Cd exposure and obesity in children, and determined the plausibility of this relationship in a controlled experimental zebrafish model. After adjusting for cigarette smoking, sex, breastfeeding and co-occurring metals (Pb and/or As), we found persistent associations between prenatal Cd exposure and increased risk of obesity from birth to age five years. Our data also suggest that these children were also more likely to have steeper growth trajectories between birth to age five years. In support of this association, we also found that zebrafish exposed developmentally to Cd at 60 ppb had estimated Cd plasma levels at seven dpf similar to those measured in the NEST cohort at term. Assuming human and zebrafish developmental landmarks scale with lifespan, the two time-points are developmentally similar stages. These fish went on to exhibit significantly higher lipid accumulation as juveniles, when compared to unexposed controls. Surprisingly, lipid accumulation plateaued at or near the onset of sexual maturity. Although similar data observations are suggested in human data, follow-up is short and sample sizes small as evidenced by the wide confidence intervals. However, if similar plateauing of obesity risk were replicated in larger studies, these findings

would support the intriguing possibility that, without postnatal exposure, Cd-associated obesity may in fact be transient.

To our knowledge, our study represents the first direct measure of association between prenatal Cd exposure and increased obesity risk in children, the results of which are supported by similar findings in an evolutionarily related model organism. Whether Cd is measured in biological materials that reflect long term chronic exposure[13-15], such as toe nails or urine or in blood, reflecting shorter term, concurrent exposure[12, 16-18], data linking elevated Cd levels to obesity related cardiometabolic diseases among adults are inconsistent[19, 20]. However, in early life, exposure to Cd is consistently associated with lower birth weight[26, 27, 72-74], although the few studies that have examined the association between prenatal Cd and growth[74] found that maternal Cd was associated with lower head circumference, height and weight. Reasons for inconsistent findings are unclear although differences in exposure dose, i.e., circulating concentration, could be a factor, which may depend on the source of exposure. Cd doses that are ingested or inhaled from contaminated air or dust are likely higher than levels in contaminated grains, which form only a fraction of the total diet. Inconsistent findings could also be due to co-exposure to other metals, which together with Cd, may have antagonistic effects, e.g., selenium. Differences could also be due to inadequate control for confounding by socioeconomic status, which in turn may influence not only dietary factors but also residence in geographic locations of higher exposure[75]. In zebrafish exposed only to Cd, limited to the human-equivalent periconceptional and early prenatal period and the elimination of socioeconomic effects, Cd exposure was associated with lipid accumulation. Whether the plateauing effect is sustained into puberty and beyond is still unknown.

Mechanisms linking low dose Cd exposure and subclinical cardiometabolic dysfunction are unclear; however, single metal analysis in adults suggests that blood Cd below reportable levels of 0.5 μg/dL was associated with elevated glucose[76-80], higher blood pressure, presumably via kidney dysfunction[81, 82], and oxidative stress[83], which depletes antioxidants[84, 85]. In autopsy specimens, higher liver Cd levels were associated with hypertension[86]. In mice and *in vitro*, early Cd exposure increased inflammation, oxidative stress, and blood pressure, doubled adipocyte numbers[87], and lowered the expression of lipid synthesis genes[88]; thus obesity could result *directly* from this increased capacity for lipid storage. In these model systems, early Cd exposure also dysregulated the release of chemokines, leptin and adiponectin[87, 88] leading to insulin resistance later in life[89]. As these chemokines are involved in appetite regulation and energy expenditure[90-92], cardiometabolic dysfunction indicators may also result *indirectly* via altered satiety responsiveness and increased caloric intake. Disentangling these possibilities will be critical in the future, to guide intervention efforts aimed at reducing Cd-related cardiometabolic dysfunction.

A major strength of our study is the ability to demonstrate in humans and in zebrafish that Cd increases lipid accumulation, leading to obesity, and associations are free from the influence of co-exposure to other metals and socioeconomic factors. However, our study had a limited sample size as evidenced by the wide confidence bands. While the sample size was adequate to demonstrate significant associations in overall analyses, we were under-powered to examine sex differences in children; Cd exposure effects may vary by sex. In addition, although prospective, children were followed from birth to age five years, and without serial specimens, the effects of postnatal exposure could not be disentangled in children. However, zebrafish that were exposed only “prenatally” had significantly higher lipid accumulation that the unexposed controls,

suggesting that postnatal exposure did not unduly influence our findings in children. Moreover, the extent to which Cd-related obesity will be maintained after age five years is unknown. Zebrafish that were followed until sexual maturity exhibited reduced lipid accumulation.

Despite these limitations, our data support the causal association between *in utero* exposure to Cd and obesity at age five years. Larger studies are required to confirm these findings and determine Cd effects vary by sex.

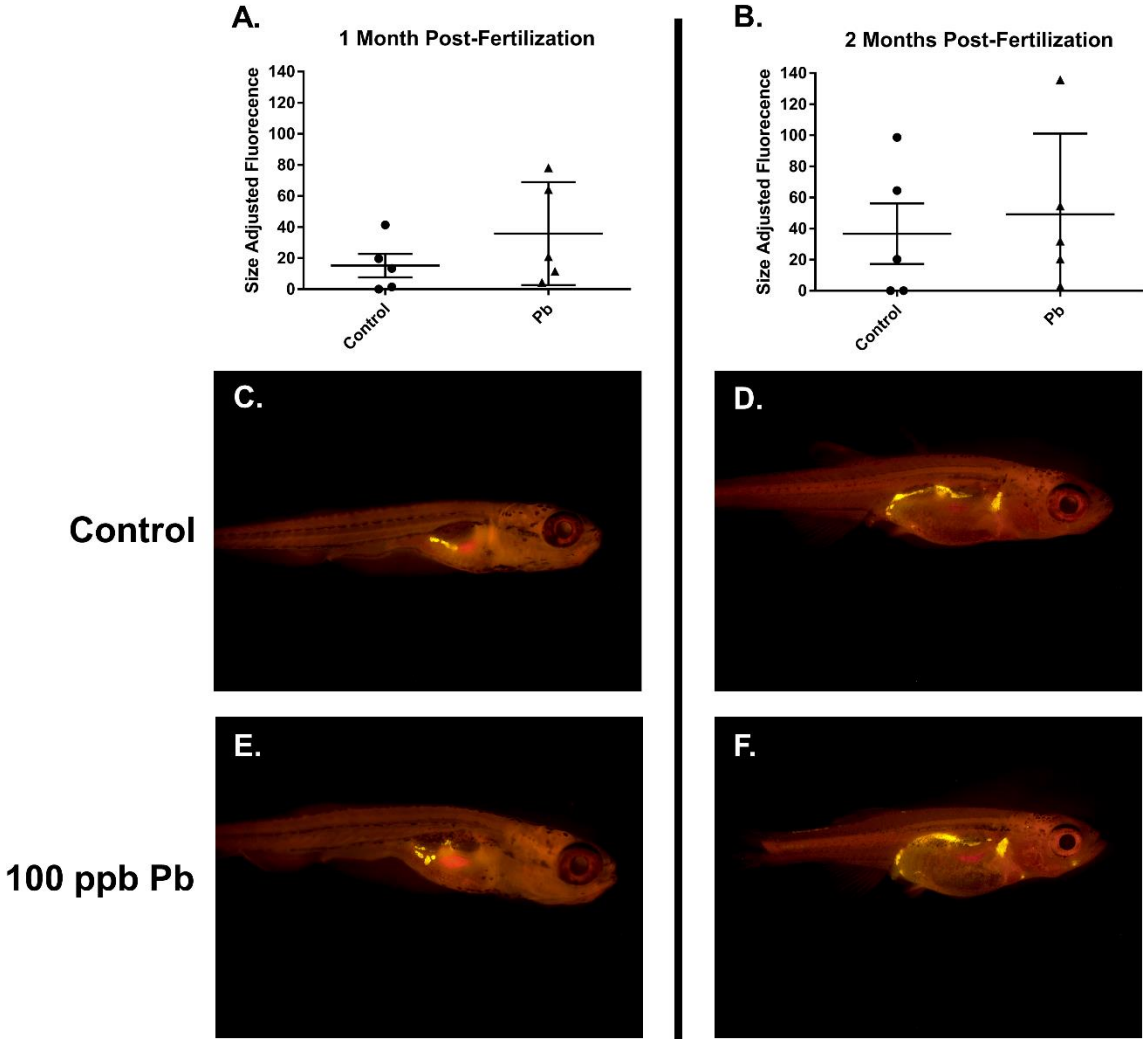

Figure s1. Developmental exposure to lead has no effect on lipid deposition in juvenile zebrafish. Nile red fluorescence was not significantly greater in zebrafish larvae exposed to 100 ppb Pb vs. water controls at one (A) and two (B) months post-fertilization ($p<0.05$). Representative live images of Nile red staining are shown for control (C, D) and Pb-exposed (E, F) zebrafish at one- and two-months post-fertilization, respectively.

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CHAPTER 3

Cadmium Promotes Mesenchymal Stem Cell-Derived Adipocyte Differentiation, Thereby Increasing Lipid Accumulation, with Concomitant Delay in Bone Formation: an *in vitro* and *in vivo* Analysis.

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Abstract

Cadmium (Cd), a ubiquitous environmental compound, affects human populations worldwide primarily through cigarette smoke and the consumption of contaminated foods. Accumulation of Cd has been associated with osteotoxicity and increased bone marrow adiposity. To understand the mechanisms behind these phenotypes, we aim to show that Cd exposure disrupts mesenchymal stem cell differentiation resulting in increased lipid accumulation and adipogenesis *in vitro*, and delayed bone formation *in vivo*. Human adipose-derived mesenchymal stem cells (hADMSCs) were exposed to 0.5 or 5 ppb Cd during adipogenic differentiation and assessed for adipogenesis using staining, and absorbance quantification of Oil Red O (ORO) extracts. To assess changes in bone formation *in vivo*, AB wild-type embryos were exposed to 0 – 100 ppb Cd from four hpf through 14 dpf and evaluated for early, intermediate, and apical stages of differentiation. Results show that exposure to 0.5 ppb Cd increases the number of hADMSCs that differentiate into adipocytes and that ORO absorbance is significantly increased beginning at 5 ppb Cd. Zebrafish exposed to 15 ppb Cd have decreased Alizarin Red staining of the notochord, pharyngeal teeth, and cleithrum compared to controls at eight dpf while those individuals exposed to 40 ppb Cd showed no evidence of staining. At 14 dpf the notochord, pharyngeal teeth, cleithrum, and opercle showed reduced mineralization in fish exposed to ≥25 ppb Cd compared to controls, but now staining was present at all concentrations tested. Cd exposure did not affect cartilage morphology at any dose at eight or 14 dpf. These data support the hypothesis that developmental exposure to Cd, at environmentally relevant levels, decreases bone mineralization, and increases adiposity perhaps by shifting the differentiation of MSCs from an osteoblast to an adipocyte lineage.

Introduction

Over the past 40 years the prevalence of obesity has increased globally, and now more people are obese than are under-weight[1]. Due to an extremely high prevalence and its association with metabolic disturbances, obesity has a significant socioeconomic impact, accounting for greater than 20% ($190 billion) of all health expenses per year in the USA[2]. Interestingly, people today who eat and exercise the same amount as people 20 years ago are still more obese than their counterparts were two decades ago, though the composition of their diet and other environmental exposures may differ[3]. Recent studies have revealed a significant association between environmental pollutants and obesity[4–7].

Cd has long been recognized as an extremely toxic and ubiquitous environmental pollutant. In the United States exposure to Cd occurs through ingestion of food and water, inhalation of particulates from ambient air or tobacco smoke, and ingestion of contaminated soil or dust[8]. The danger of Cd is that it has an extremely long half-life in the body of greater than 15 years[9] and exposure has well-established effects on bone including increased risk of bone fractures[10–12] and osteoporosis[12–15], and decreased bone mineral density[16–19]. Similar effects have been seen in both mammalian and fish model systems but very few have looked at the effects of Cd during development[20–24].

Data from human studies on the interaction between Cd exposure and obesity are rather contradictory, with studies showing that exposure to Cd is correlated with both increases and decreases in the incidence of obesity. In particular, it has been demonstrated that blood Cd levels are associated with higher body mass index (BMI), and waist and hip circumference in girls aged 8–15 years[25]. A detailed analysis of data from the NHANES 1999–2002 study revealed a direct correlation between BMI, waist circumference and urinary Cd levels[26]. On the other hand, in

postmenopausal Swedish women erythrocyte Cd content showed a slightly but significantly inverse association with BMI[27], whereas a study of middle-aged residents of abandoned metal mines in South Korea revealed a significant negative association between blood and urinary Cd levels and BMI in men but no significant association in women[28]. Multiple regression analysis performed using data from NHANES 1999–2008 found a significant inverse association between urinary Cd and BMI values in children, teens, and smoking adults, but not in the non-smoking adults[29]. The existing contradictions between the results of the above-mentioned studies may be at least partially due to the differences in Cd exposure levels as well as the use of different markers for Cd exposure, such as urine, blood, and hair. However, laboratory studies consistently show that Cd may alter adipose tissue physiology and induce an obesity-associated metabolic profile, along with an increased production of fat in bone marrow[21,30]. Considering that MSCs transition from multipotent to differentiated cell types, including chondrocytes (cartilage), osteocytes (bone), and adipocytes (fat), Rodríguez and Mandalunis proposed that the increase in tibial yellow bone marrow caused by Cd was the result of either inhibition of the differentiation of mesenchymal cells to osteoblasts, instead favoring adipocytes, or through the promotion of osteoclastogenesis although empirical data are limited[21]. Although Cd has been shown to affect bone development *in vivo*, little is known about how it affects osteogenic or adipogenic differentiation of MSCs. In this study, we examined the effects of Cd exposure on hADMSCs during adipogenesis, and zebrafish bone formation (Fig. 1) and gene expression. Our results show that developmental Cd exposure decreases zebrafish bone mineralization, and increases adipocyte development and lipid accumulation in hADMSC exposed to Cd during adipogenic differentiation.

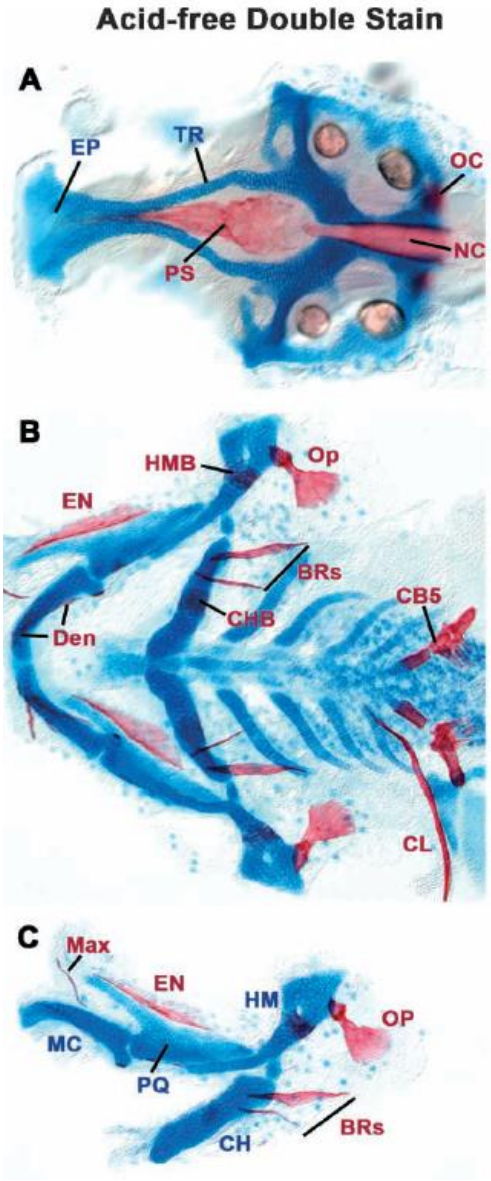

Figure 1: Acid-free double staining in larval zebrafish. Adapted from Walker, M., and Kimmel, C. (2007). A two-color acid-free cartilage and bone stain for zebrafish larvae. Biotechnic & Histochemistry 82, 23–28. (A) Dorsal view of the neurocranium. (B) Ventral view of the pharyngeal skeleton. (C) Lateral view of the jaw and jaw support. Bones: PS, parasphenoid; OC, occipitals; NC, notochord; Max, maxilla; Den, dentary; EN, entopterygoid; BRs, branchiostegal rays; OP, opercle; HMB, hyomandibular bone; CHB, ceratohyal bone; CB5, ceratobranchial 5, CL cleithrum. Cartilages: EP, ethmoid plate; TR, trabeculae; MC, Meckel's Cartilage; PQ, palatoquadrate; HM, hyomandibular region of the hyosymplectic cartilage; CH, ceratohyal cartilage.

Materials and Methods

Animal husbandry

Wildtype (AB strain) zebrafish were maintained in the NC State University Zebrafish Core Facility according to standard protocols[31]. All work involving zebrafish was approved by the NC State Animal Care and Use Committee.

Chemicals

A stock solution of cadmium chloride (99.99% purity; Sigma-Aldrich, St. Louis, MO) was dissolved in reagent-grade (Picopure®) water at 10 parts-per-thousand (ppt) and stored at -20˚C in 1.5 mL polypropylene tubes. Substocks were made in reagent-grade (Picopure®) water at 10 - 100 parts-per-million (ppm; 1000x) and stored at room temperature in 1.5 mL polypropylene tubes. Substocks were made in cell growth media at 500 ppb (100x) and stored at 4˚C in 1.5 mL polypropylene tubes. A stock solution of Rosiglitazone ([Rosi], ≥ 98% purity; Sigma-Aldrich, St. Louis, MO) was made in DMSO at 10 mM and stored at -20˚C in 1.5 mL polypropylene tubes. Substocks were made in DMSO at 100 μM (1000x) and stored at 4˚C in 0.3 mL polypropylene tubes.

hADMSC cell culture

Human ADMSCs from a 33-year-old Caucasian female (American Type Culture Collection, PCS-500-011, Manassas, VA) and from a 55-year-old Caucasian female (93831, a gift from Seth Kullman) were cultured at an early passage (P). Frozen hADMSCs at P5 were revived and cultured in growth medium (GM) comprised of minimal essential medium, α-modification (MEM-α, GE Healthcare, Chicago, IL) supplemented with 10% fetal bovine serum

(FBS, Rocky Mountain Biologicals, Missoula, MT), 2 mM L-glutamine (≥99% purity; Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin, and 100 µg/mL streptomycin (Genesee Scientific, San Diego, CA) and incubated at 37°C and 5% CO_2. Medium changes were performed every three to four days and cells were passaged at ~80% confluence. For harvesting, the cultures were treated with 0.05% Trypsin-EDTA (Gibco, Gaithersburg, MD) at 37°C and 5% CO_2 for 3 minutes (sufficient time to obtain a total suspension of the culture). Cells were then transferred to GM and centrifuged at 300 ×g for 5 minutes. Cell pellets were subsequently resuspended by pipetting, and plated in 12-well tissue culture-treated plates (Genesee Scientific, San Diego, CA) at a density of $1.5x10^4$ cells/cm^2 in GM. After 48 hours, the media was changed to adipogenic differentiation medium (ADM) comprised of α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, 1 µM dexamethasone (Sigma-Aldrich, St. Louis, MO), 5 µg/mL insulin (human; Sigma-Aldrich, MO), 100 µM indomethacin (Sigma-Aldrich, St. Louis, MO), and 500 µM isobutylmethylxanthine (IBMX, Sigma-Aldrich, St. Louis, MO) containing either 0, 0.5, or 5 ppb Cd, or 0.1 µM Rosi. Media was changed every 48 hours for four days after, after which the media was changed to adipogenic maintenance medium (AMM) comprised of GM supplemented with 5 µg/mL insulin (human; Sigma-Aldrich, St. Louis, MO) containing either 0, 0.5, or 5 ppb Cd, or 0.1 µM Rosi. Media was changed every three to four days for 20 days. Afterwards, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS at 4˚C and then assessed for number of adipocytes and lipid accumulation.

Exposures

Zebrafish embryos were collected immediately after spawning and exposed to 0 - 100 ppb Cd in 0.5X E2 media (7.5 mM NaCl; 250 µM KCl; 500 µM $MgSO_4$; 75 µM KH_2PO_4; 25 µM Na_2HPO_4; 500 µM $CaCl_2$; 350 µM $NaHCO_3$) from four hpf through 14 dpf at a density of 10 embryos/mL. This concentration range was selected as it represents the range observed in human populations exposed to Cd-polluted environments[8,32–34]. The media was replaced daily and feeding began at five dpf.

Adipogenic assays measuring lipid accumulation

Cells were stained with Oil Red O (ORO, Sigma-Aldrich, St. Louis, MO) and subsequently destained to assess adipogenesis in hADMSCs following ADM-AMM culture. Fixed cells were washed with 60% isopropyl alcohol and then stained for 10 minutes in filtered ORO staining solution composed of three parts 0.35% ORO in 100% isopropyl alcohol to two parts reagent-grade (Picopure®) water. Wells were washed with ddH_2O and imaged under a Leica dissecting microscope at 10x magnification using the Leica Application Suite (version 4.8.0). Images were analyzed using Fiji (ImageJ, version 1.52n)[35] to subtract the background and count cells using the automatic particle sizing method described by Ruzicka[36]. To quantify lipid accumulation, wells were aspirated, allowed to dry, and the stain extracted via addition of 100% isopropyl alcohol. Absorbance of the extract was measured at 500 nm.

Whole-mount histological staining for bone and cartilage

Larvae were euthanized at seven and 14 dpf with 1 mg/L solution of phosphate-buffered Tricaine (Western Chemical Inc., Ferndale, WA) and fixed overnight at 4˚C in 4%

paraformaldehyde in PBS. Following fixation, larvae were dehydrated with 33% and 66% methanol and stored at -20˚C prior to staining. A subset of 10-12 individuals was stained for mineralized bone with 0.05% Alizarin Red (Sigma Aldrich, St Louis, MO) in 70% ethanol overnight. Post staining, individuals were washed with ddH_2O, followed by a 60-minute incubation in 2% H_2O_2 in 0.5% KOH, and washed twice in 0.25% KOH. Samples were then digested in 0.05% trypsin (Difco™ BD Biosciences, San Jose, CA) dissolved in 30% saturated sodium borate for 20 minutes at room temperature. Following tissue digestion, samples were washed with 0.25% KOH, and cleared in a graded series of 25%, 50%, and 75% glycerol in 0.1% KOH, and stored at 4˚ C. Samples were then imaged under a Leica dissecting microscope at 80x magnification.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8.1.0)[38]. All data were analyzed for normality before statistical analysis using both the D'Agostino & Pearson normality test[39] and the Shapiro-Wilk normality test[40]. The significance of the lipid extracts and ORO positive cells was determined by one-way ANOVA followed by Holm-Sidak's multiple comparisons test[41].

Results

Adipogenic assays measuring lipid accumulation

Based on the ability of Cd to increase lipid accumulation and body weight *in vivo*, shown in chapter 2, the following experiments were conducted to determine whether Cd-exposure increases the adipogenic potential of hADMSCs during adipogenic differentiation. hADMSCs were exposed to Cd or Rosi in ADM and AMM followed by staining with ORO to assess lipid formation (Fig. 2a) and adipocyte development (Fig. 2b). By 24 days post-induction, positive controls displayed positive ORO staining (Fig. 2c) while Cd-exposed cells demonstrated an overall increase in the number of ORO positive adipocytes (Fig. 2d) compared to unexposed controls. Image analysis of the entire well using FIJI shows that this increase in ORO positive cells appears to be concentration-dependent and that exposure to 5 ppb Cd resulted in a similar number of ORO-positive adipocytes compared to the Rosi control (Fig. 2b). Additionally, relative ORO absorbance (Fig. 2a) in isopropanol extracts shows significant increases in both the 5 ppb Cd ($p < 0.01$) and the Rosi control ($p < 0.001$) compared to the unexposed controls. These results show that during hADMSC differentiation, Cd acts in a similar phenotypic manner to the known obesogen Rosiglitazone.

Whole-mount histological staining for bone and cartilage

Preliminary range-finding experiments with zebrafish exposed to 0, 1, or 100 ppb Cd (n=10) from four hpf to seven dpf were performed to assess bone and cartilage development (Fig. 3). These concentrations were chosen as they fall below, within, and above the range of likely human fetal exposure (0.15 – 16 ppb Cd)[32,42]. At seven dpf the larvae were fixed and

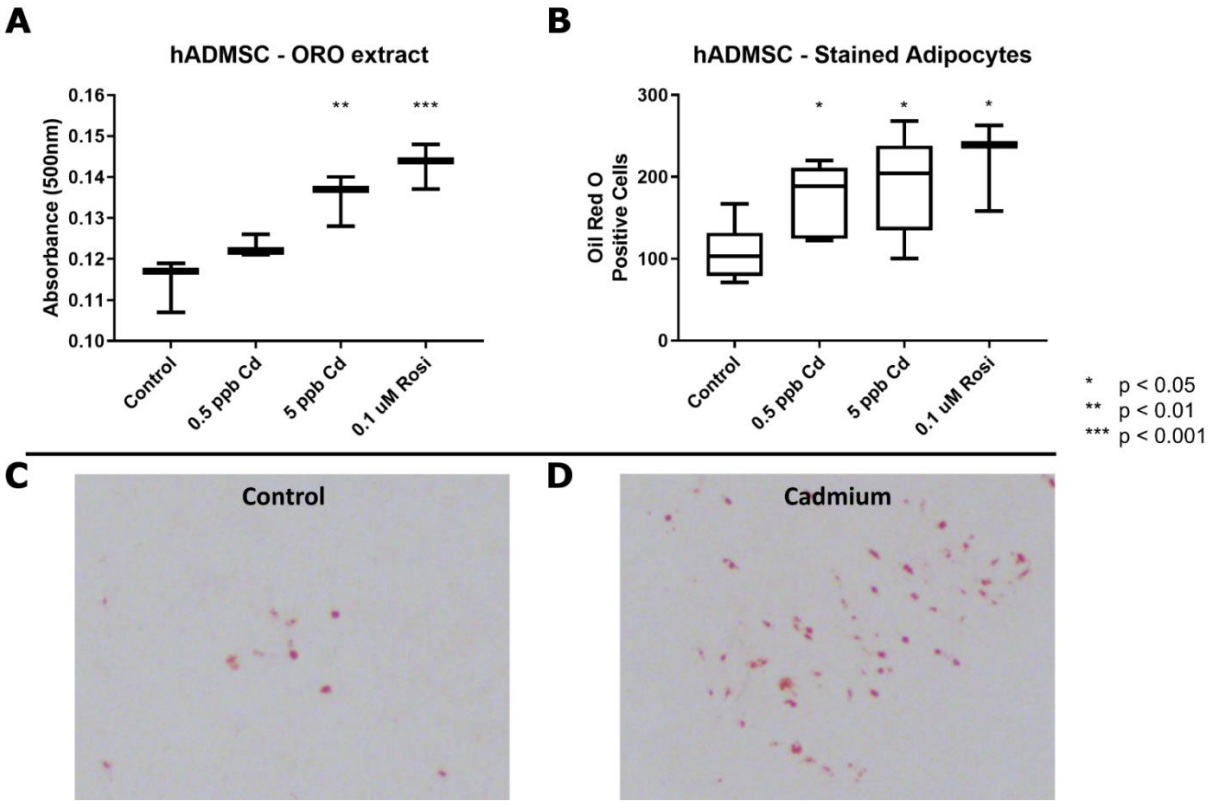

Figure 2: Cd-exposure during adipogenesis increases adiposity in hADMSCs. (A) Absorbance of lipid extracts from hADMSCs exposed to Cd or Rosiglitazone during adipogenic differentiation. (B) Quantification of the number of ORO positive hADMSCs following Cd or Rosiglitazone exposure during adipogenic differentiation. (C) Representative image of unexposed ORO positive hADMSCs on day 24 of adipogenic differentiation. (D) Representative image of ORO positive hADMSCs exposed to 5 ppb Cd on day 24 of adipogenic differentiation.

stained with Alcian Blue to assess cartilage morphology and with Alizarin Red to investigate bone mineralization (Fig. 3). Control and 1 ppb Cd-exposed individuals displayed positive Alcian Blue staining of Meckel's (MC), palatoquadrate (PQ), ceratohyal (CH), hypobranchial (HB), ceratobranchials (CB), ethmoid plate (EP), trabeculae (TR), and notochord cartilage (NC) (Fig. 3). Alizarin Red staining was observed in the otoliths (OT), pharyngeal teeth (PT), and along the anterior position of the notochord (NC) (Fig. 3). Individuals exposed to 100 ppb Cd also displayed positive Alcian Blue staining of the major craniofacial cartilage elements; however, there was a complete lack of bone mineralization as indicated by no observed Alizarin

Red staining (Fig. 3). This dramatic effect observed at the highest concentration shows that developmental exposure to Cd results in a marked attenuation in zebrafish bone mineralization but does not interfere with cartilage formation.

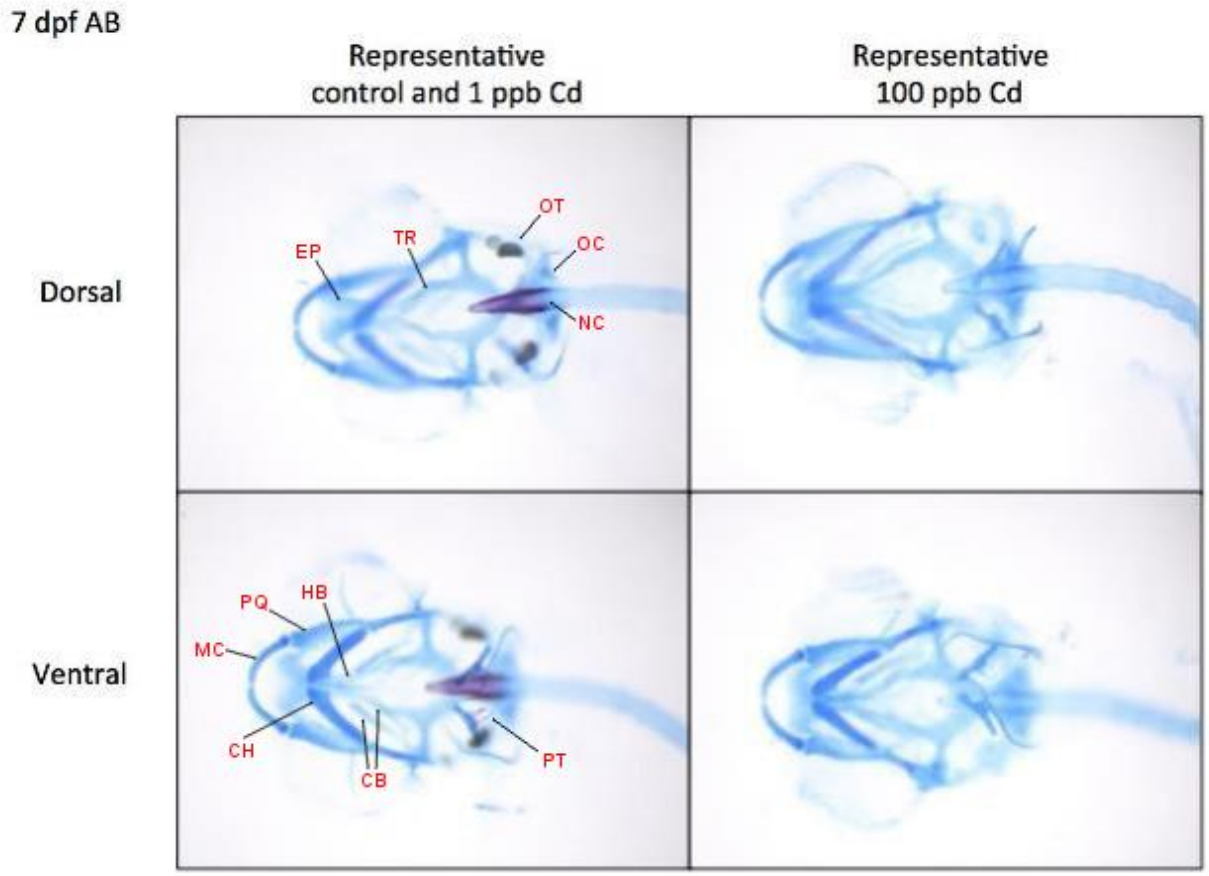

Figure 3: Cd delays bone calcification at seven dpf. Representative dorsal and ventral images of Alizarin Red (Bone, purple) and Alcian Blue (Cartilage, blue) stained zebrafish larvae at seven dpf following exposure to 0, 1, or 100 ppb Cd from four hpf through seven dpf. OT – Otolith, OC – Occipitals, NC – Notochord, PT – Pharyngeal teeth, MC – Meckel's, PQ – Palatoquadrate, CH – Ceratohyal, HB – Hypobranchial, CB – Ceratobranchials, EP – ethmoid plate, and TR – Trabeculae.

Concentration-response staining for bone

To confirm the concentration-dependent relationship between developmental Cd exposure and bone mineralization we exposed zebrafish embryos to 0, 15, 25, 30, 35, and 40 ppb

Cd from four hpf through 14 dpf. At seven dpf, larvae (n=10) were fixed and stained with Alizarin Red. These results, shown in figure 4, show a concentration-dependent decrease in Alizarin Red staining at all concentrations with no staining observed at 40 ppb Cd. Detailed assessment of the individuals exposed to 15 ppb Cd shows a decrease in staining of the notochord, pharyngeal teeth, and cleithrum (Fig. 4). It is also worth noting a concentration-dependent decrease in otolith size beginning at 25 ppb Cd (Fig. 4). This will be discussed in further detail in chapter 4. At 14 dpf, larvae (n=10) were once again assessed using Alizarin Red staining (Fig. 5). At this time, we observed that mineralization was present at all concentrations but was notably decreased beginning at 25 ppb Cd with a maximal decrease at 40 ppb Cd in the notochord, pharyngeal teeth, cleithrum, and opercle (Fig. 5). These results show that developmental Cd exposure is able to impair bone mineralization at the lowest concentration tested (15 ppb Cd), a level of exposure that is equivalent to the upper end of fetal exposure (0.15 – 16 ppb Cd)[32,42], and that this decrease in mineralization is persistent.

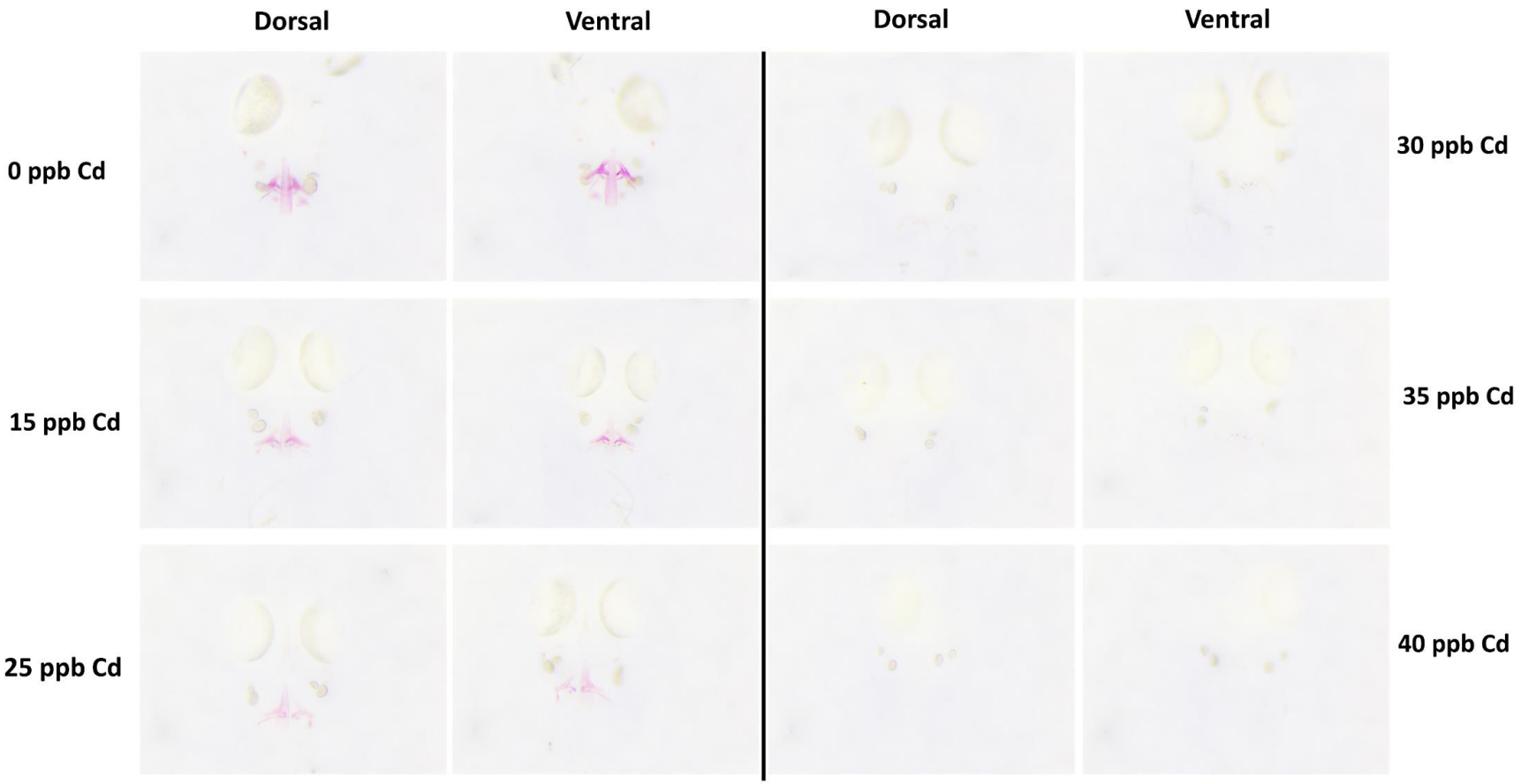

Figure 4: Cd-exposure causes a concentration-dependent decrease in mineralization at eight dpf. Representative dorsal and ventral images of Alizarin Red (Bone, purple) stained zebrafish larvae at eight dpf following exposure to 0 to 40 ppb Cd from four hpf.

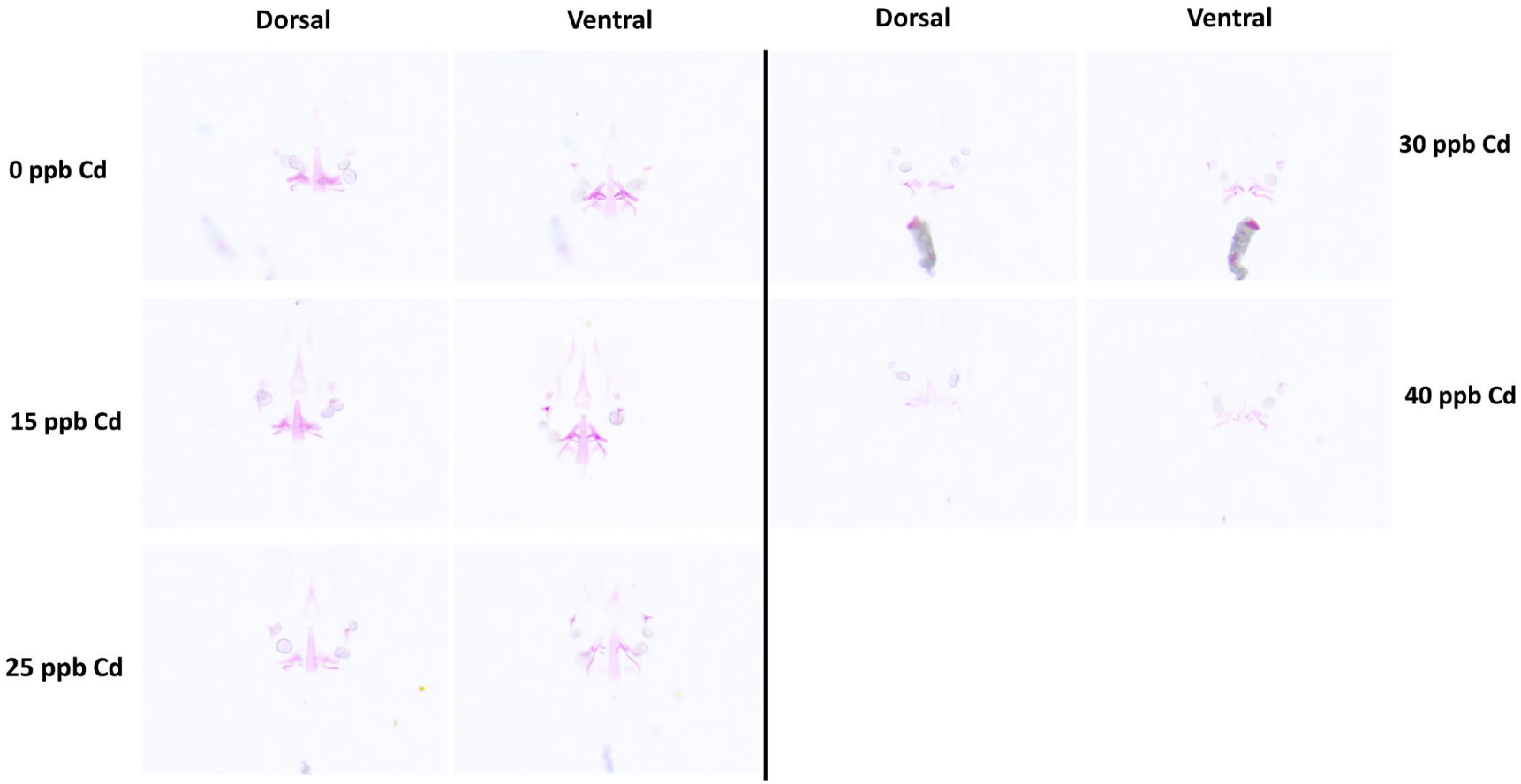

Figure 5: Concentration-dependent decrease in mineralization caused by Cd persists after 14 days of chronic exposure. Representative dorsal and ventral images of Alizarin Red (Bone, purple) stained zebrafish larvae at 14 dpf following exposure to 0 to 40 ppb Cd from four hpf.

Discussion

In this chapter we have shown that hADMSCs exposed to Cd at levels found in human populations are capable of inducing significant increases in both the number of differentiated adipocytes as well as the amount of accumulated lipid. We observed that in zebrafish developmentally exposed to 100 ppb Cd, there was a complete absence in bone mineralization at seven dpf compared to low concentration Cd or unexposed controls. This loss of bone mineralization was not the result of losing major craniofacial cartilaginous structures. The Cd-induced decrease in bone mineralization is concentration-dependent, with the notochord, pharyngeal teeth, and cleithrum all exhibiting loss of mineralization at 15 ppb Cd. Furthermore, following 14 days of chronic exposure to Cd we observed mineralization at all concentrations tested, whereas larvae exposed to 15 ppb Cd were indistinguishable from controls. While all concentrations now showed positive Alizarin Red staining, there were still significant decreases compared to unexposed controls and this decrease was more significant as the concentration increased. We proposed that Cd exposure disrupts adipogenic differentiation and increases lipid accumulation *in vitro*, and delays bone formation *in vivo*.

To support this hypothesis, we have shown that Cd decreases bone mineralization, *in vivo,* in a concentration-dependent manner following developmental exposure, and increases the number of MSC's that differentiate into adipocytes. This is concerning as blood Cd concentrations have been reported as high as 9.17 and 30.9 ppb in the U.S.[43] and internationally[32] in recent years, respectively. The placenta may partially protect a developing fetus from exposure as cord blood Cd concentrations have been shown to be approximately half as high as those found in maternal blood[44–46]. While a study conducted in Cincinnati found a mean Cd concentration of 19 ppb in human milk and it is estimated that between 5-10% is absorbed by newborns[47,48]. These data highlight that fetal / newborn MSC's are likely exposed to Cd at

concentrations very similar to those we have demonstrated have the ability to increase adipocyte differentiation and lipid accumulation. Recent developmental and early life studies show that exposure to Cd increases the total fat mass and circulating lipids in male mice[20], and decreases bone volume while increasing bone marrow fat in juvenile rats[21]. Our data support these *in vivo* findings and provide mechanistic insights on how low concentration Cd exposure is capable of increasing MSC adipogenic differentiation. Considering that our experiments were conducted in hADMSCs and that Cd exposure increases bone marrow fat, it appears that Cd may be able to enhance adipogenesis independent of the specific MSC niche. Further, our cell culture lines were derived from female donors and the animal model data shows increased susceptibility only in males suggesting that while female MSCs are vulnerable to Cd-induced adipogeneses *in vitro*, sex-specific factors such as differential DNA methylation[48], hormonal differences, and gut microbiota[20] may all play a role. Therefore, future studies should be conducted in MSC's derived from both males and females.

The toxic effects of Cd exposure on bone have been well established in epidemiological studies, and include increased risk of bone fractures[10–12] and osteoporosis[12–15], and decreased bone mineral density[16–19]. Similar effects have been observed in prenatal, postnatal, juvenile, adult, and aged mammalian models exposed Cd[20–23]. A recent study by Tarasco et al. showed that zebrafish developmentally exposed to 5 ppb Cd have decreased mineralization of the opercle, increased incidence of skeletal deformities, and decreased expression of bone marker genes including *spp1* and *sparc* at 20 dpf[24]. Our data show that Cd exposure is able to decrease the mineralization of a number of bones including the notochord, pharyngeal teeth, cleithrum, and opercle without affecting cartilage formation. In Zebrafish, *runx2* plays a dominant role in the transition of MSCs within mesenchymal condensations of cells undergoing active

chondrogenesis. Flores et al. showed that *runx2b*, but not *runx2a*, plays a pivotal role in this process and that while *runx2a* is dispensable in early craniofacial cartilage development, *runx2b* is necessary during these stages[49]. Later work using *in situ* hybridization shows that before 36 hpf there is dispersed expression of both *runx2a* and *runx2b* but by 48 hpf expression is mostly contained around the developing skeleton where they both play a key role in the immature osteoblast[50]. Runx2, induces the expression of, or activates the promoters of major bone matrix protein genes, including *Col1a1, Col1a2, Spp1, Ibsp/BSP, Bglap2, Fn1/fibronectin, Mmp13,* and *Tnfrsf11b/Opg*. The extracellular matrix (ECM) protein Spp1is found in osteoblasts and mineralized bone matrix[51], which enhances osteoblastic differentiation and proliferation[52,53] facilitating bone formation and resorption[54]. While more work is needed, and our study did not assess osteoblast numbers directly or osteogenic gene expression, these data support the hypothesis that developmental Cd exposure decreases bone mineralization and increases adiposity by shifting the differentiation of MSCs from an osteoblast to an adipocyte lineage.

Future studies are needed to assess the expression of the major bone development genes (*runx2, spp1, col1a1, bglap),* to determine the effects of Cd exposure on osteogenesis in both hADMSC and human bone derived mesenchymal stem cells (hBDMSC), assess how Cd exposure affects adipogenesis in hBDMSC, and evaluate the effects of Cd exposure on adipogenic and lipogenic gene expression *in vitro*.

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CHAPTER 4

Developmental Cadmium Exposure Disrupts Vestibular Calcium Channels Interfering with Otolith Formation and Inner Ear Function.

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Abstract

Dizziness or balance problems are estimated to affect approximately 3.3 million children aged three to 17 years. These disorders develop from a breakdown in the balance control system and can be caused by anything that affects the inner ear or the brain, including exposure to environmental toxicants. One potential environmental toxicant linked to balance disorders is cadmium (Cd), an extremely toxic metal that occurs naturally and as a toxic byproduct of industrial processes. Cd is associated with balance and vestibular dysfunction in adults exposed occupationally to Cd, but little is known about the developmental effects of low concentration Cd exposure. Our results show that zebrafish exposed developmentally to 10 - 60 ppb Cd from four hpf to seven dpf exhibit abnormal behaviors, including pronounced increases in auditory sensitivity and circling motions, both of which are linked to reductions in otolith growth. Pharmacological intervention shows that agonist-induced activation of the P2X in the presence of Cd is able to restore otolith size and minimize behavioral abnormalities. In conclusion, Cd-induced ototoxicity is linked to vestibular-based behavioral abnormalities and auditory sensitivity following developmental exposure and P2X channel function are associated with these defects.

Introduction

In the United States 3.3 million children (5.3%) suffer from dizziness and balance disorders[1]. These vestibular disorders can cause life altering disability and rank among the most common reasons for referral to neurologists and otolaryngologists[1]. Vestibular disorders develop from a breakdown in the balance control system and can be caused by factors that affect the inner ear or the brain, including environmental and pharmaceutical exposures, ear infections, and head trauma[1–5]. This intricate balance control system includes both visual and vestibular inputs, together with the central nervous system, to detect and integrate both the magnitude and direction of head movement[4]. The vestibular system of vertebrates consists of three semicircular canals harboring sensory epithelia that respond to rotational acceleration, and two gravity receptor organs, the utricle and saccule, which sense linear acceleration and gravity (Fig. 1a and 1b)[2,6]. The utricle and saccule house bio-crystals composed of calcium carbonate ($CaCO_3$) and proteins[2,6], which lie above the cilia of hair cells of the sensory epithelium (macula). The correct formation and anchoring of these bio-crystals is essential for optimal vestibular function, balance, and the detection of sound (Fig. 1c and d) [2,7,8].

While the factors that cause dizziness and balance disorders in children are poorly understood, recent epidemiological studies have uncovered evidence of balance dysfunction, complaints about equilibrium, and slowing visuomotor function in adults occupationally exposed to heavy metals, including cadmium (Cd)[4,9], a non-essential transition metal widely used in the production of batteries, solar panels, pigments, plastic stabilizers, and the production of other metals[10]. Oxidized Cd typically enters the body through contaminated food and water as well as through inhalation of polluted air and cigarette smoke[10,11]. Cd has been shown to accumulate in the cochlea of rats, damaging the hair cells and leading to hearing loss[10]. Interestingly, treatment

with antioxidants results in significant protection against the ototoxic effects of Cd [7].

Additionally, Cd is associated with impaired neurodevelopment[12] and altered social and escape behavior in fish models[13–15].

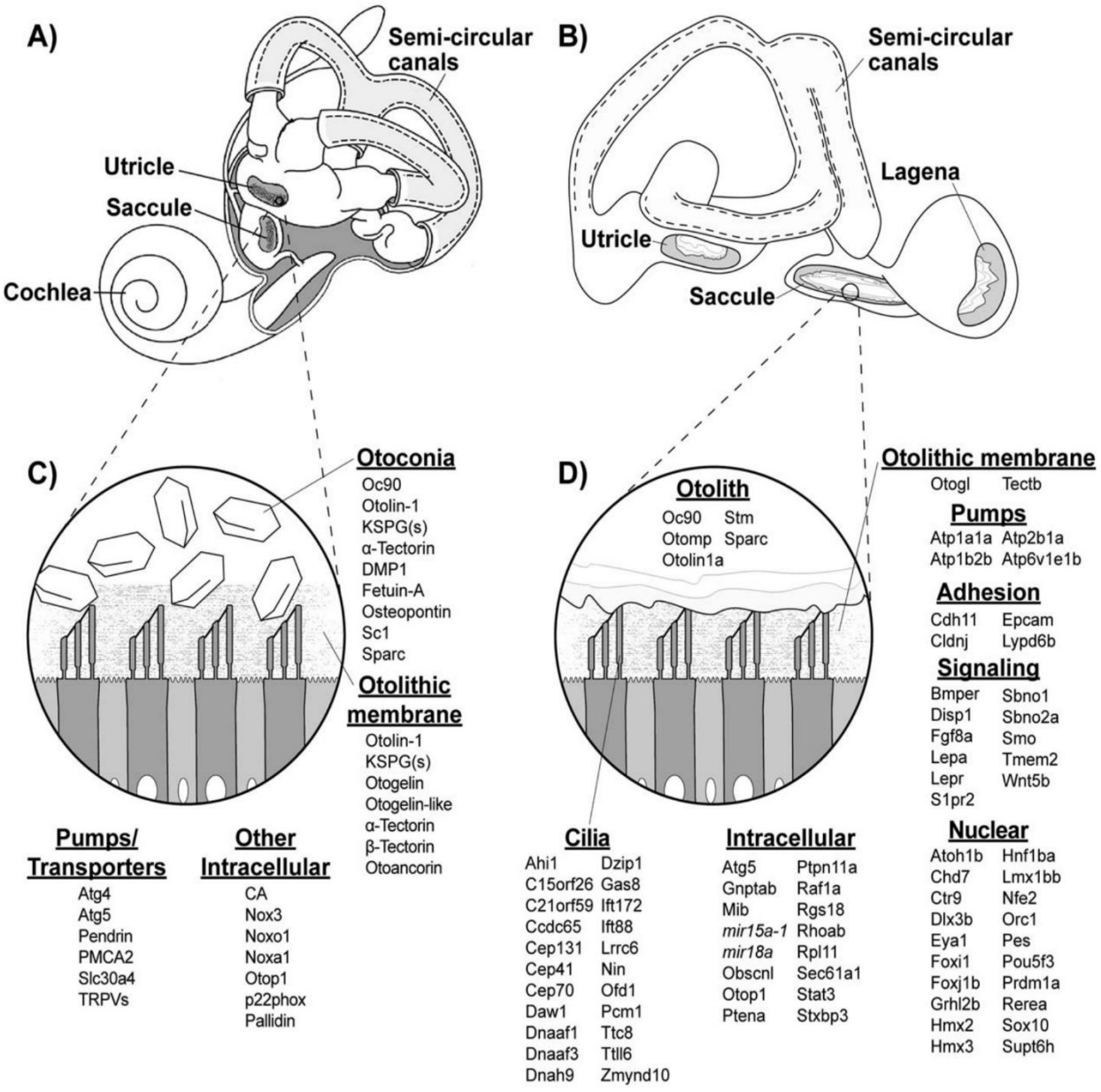

Figure 1: An illustration of the mammalian and zebrafish inner ear. Reproduced from Lundberg et al. (2015). (A) Mammalian inner ear. (B) Zebrafish inner ear. (C) Zoomed-in illustration of Otoconia in the mammalian vestibular space. (D) Zoomed-in illustration of Otoliths is the zebrafish vestibular space.

Recent epidemiological studies and NHANES data from 2009 - 2016 shows that in the U.S blood cadmium levels range from 47 to 775 ng/g of body weight and are similar to the range of 158 to 667 ng/g of body weight published by IARC in 1993[11,16–22]. During gestation the placenta may act as a partial barrier to fetal exposure as Cd concentrations have been found to be approximately half as high in cord blood as in maternal blood in several studies including both smoking and nonsmoking women[23–25]. When looking at recent studies from developed countries we see that in those with measurable cord blood Cd, the newborn total body burden ranges from 67 to 450 ng/g with the caveat that these values were calculated using the Kjellström and Nordberg toxicokinetic model for Cd, which was developed for adults[17,26,27]. It is also worth noting that a study performed in Cincinnati, Ohio, observed that Cd in human milk had a mean concentration of 19 ppb[28]. In chapter 1 we showed that zebrafish exposed to 60 ppb Cd have a larval total body burden of 386 ng/g, which falls in the middle of the range for maternal total body burden and close to the upper end of the range observed in newborns and was therefore the highest concentration tested in this study.

While Cd is known to impair neurodevelopment and cause vestibular damage, little is known about how these alterations to the vestibular system influence behavior. To answer this question we utilized the zebrafish (*Danio rerio*), an increasingly valuable vertebrate model system in toxicology owing to its genetic conservation with humans, its transparency and experimental tractability, and the availability of abundant embryos from a single spawning event [29,30]. In addition, there is a high degree of anatomical conservation in the inner ear between mammals and zebrafish, making zebrafish a robust tool to identify the mechanisms underlying environmentally induced ototoxicity[2,31].

Materials and Methods

Animal husbandry

Wildtype (AB strain) zebrafish were maintained in the NC State University Zebrafish Core Facility according to standard protocols[32]. All work involving zebrafish was approved by the NC State Animal Care and Use Committee.

Chemicals

A stock solution of cadmium chloride (99.99% purity; Sigma-Aldrich, St. Louis, MO) was dissolved in reagent-grade (Picopure®) water at 10 ppt and stored at -20˚C in 1.5 mL polypropylene tubes. Substocks were made at, 10 - 60 ppm (1000x) and stored at room temperature in 1.5 mL polypropylene tubes. N-Acetyl Cysteine ([NAC],>99% purity; Sigma-Aldrich, St. Louis, MO) was dissolved in reagent-grade water at 25 mg/mL and stored at -20˚C in 1.5 mL polypropylene tubes. A calcium chloride stock was prepared at 0.5 M and stored at room temperature. ([$CaCl_2$],99% purity; Sigma-Aldrich, St. Louis, MO). Stock solutions of 2-aminoethyl diphenylborinate ([2-APB],97% purity; Sigma-Aldrich, St. Louis, MO), NF 023 hydrate ([NF023],98% purity; Sigma-Aldrich, St. Louis, MO), and 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt ([Bz-ATP],93% purity; Sigma-Aldrich, St. Louis, MO) were prepared at a concentration of 10 mM and stored at -20˚C in amber glass.

Exposures

Zebrafish embryos were collected immediately after spawning and exposed to 0 - 60 ppb Cd in 0.5X E2 media (7.5 mM NaCl; 250 µM KCl; 500 µM $MgSO_4$; 75 µM KH_2PO_4; 25 µM

Na_2HPO_4; 500 µM $CaCl_2$; 350 µM $NaHCO_3$) from four hpf through seven dpf at a density of 10 embryos/mL (Fig. 2). This concentration range was selected as it represents the range observed in human populations exposed to cadmium-polluted environments, as stated above[11,33–35]. The media was replaced daily and feeding began at five dpf. To assess the effects of Cd on otolith growth and nucleation we exposed zebrafish larvae to 40 ppb Cd from four hpf to seven dpf and measured the diameter of the saccule otolith daily as described below.

Zebrafish were co-exposed to 40 ppb Cd and 1 mg/L NAC or 2.5 mM $CaCl_2$ from four hpf through seven dpf with daily media changes to assess the impact of NAC and Calcium on the rescue of otolith size and behavior. Startle sensitivity and rotational behavior were assessed at five dpf while otolith diameter was assessed at seven dpf. To determine the effects of Cd on inner ear calcium channels zebrafish were co-exposed to 40 ppb Cd and 1 µM 2-APB, 100 µM NF023, or 100 µM Bz-ATP from four hpf to five dpf with daily media changes. Otolith diameter was assessed at five dpf.

Scanning electron microscopy

At seven dpf, larval zebrafish were euthanized. Half the larvae were dehydrated in a graded ethanol series to 100% before the oval window was teased apart using 1 µm tungsten dissecting needles (Roboz Surgical, Gaithersburg, MD) to reveal the inner ear and its associated otoliths. All larvae were then fixed in 3% glutaraldehyde in 0.1M NaPO4 buffer, pH 7.4 at 4˚C. Larvae were transferred to microporous specimen capsules (Structure Probe Inc., West Chester, PA) for processing. Larvae were washed in three changes of phosphate buffer and dehydrated in a graded ethanol series to 100% (as described above) before critical point drying in liquid CO_2 (Tousimis Research Corporation, Rockville, MD). Larvae were placed on carbon tabs (Ladd

Research Industries, Williston, VT) and sputter coated with Au/Pd using a Hummer 6.2 sputter system (Anatech USA, Union City, CA). Samples were viewed on a JEOL JSM-5900LV at 10 kV) (JEOL USA, Peabody, MA) (Fig. 2b & 2c).

Otolith imaging and measurements

Light microscopy images of otoliths were taken using a Leica dissecting microscope at 80x magnification. Zebrafish larvae (n=6-10) were anesthetized in a 0.4 mg/L solution of phosphate-buffered Tricaine (Western Chemical Inc., Ferndale, WA) in 0.5X E2 media until no movement was observed. Once anesthetized, images of zebrafish oval windows were taken using the Leica Application Suite (version 4.8.0) (Fig. 2d & 2e). The diameter of the otoliths was determined across the longest axis using the GNU Image Manipulation Programs protractor tool (version 2.8.18)[36].

Behavior assays and analysis

Rotational behavior was assessed using a DanioVision™ box with EthoVision® XT software (Noldus; Leesburg, VA). Behavior for all exposures was conducted at five dpf after zebrafish larvae were arrayed in a six-well plate at a density of eight larvae per well. Plates were placed in the DanioVision™ box and larvae were allowed to acclimate in the dark for at least 30 minutes. Following acclimation, larval rotation response to three 10-minute dark-light cycles was measured manually.

Startle response experiments were performed using five dpf larvae and analyzed using FLOTE software as described previously[37–40]. The C-startle responses were measured using defined kinematic parameters (latency, turn angle, duration and maximum angular velocity).

Startle sensitivity was determined by measuring startle frequency using a 60 sec stimulus assay with 20 sec inter-stimulus intervals and 10 trials at each of the following intensities: -8.02 dB, 0.62 dB, 5.3 dB, 10.9 dB, 17.7 dB, 25.9 dB. A startle sensitivity index for both the short latency (SLC) and long latency C-startle (LLC) was calculated for each larva by measuring the area under the curve of startle frequency versus stimulus intensity using GraphPad Prism software[41]. All stimuli were calibrated with a PCB Piezotronics accelerometer (#355B04) and signal conditioner (#482A21), and voltage outputs were converted to dB using the formula dB = 20 log (V / 0.775).

To confirm that the effects observed were due to vestibular dysfunction, we performed lateral line ablation using neomycin[42]. Larvae (n=45), exposed to 40 ppb Cd along with unexposed controls were placed in 15 mL polypropylene tubes with 5 mL of 0.5X E2 media with and without 50 µM neomycin (Sigma-Aldrich, St. Louis, MO) and incubated for one hour at 28.5°C. Larvae were washed four times in embryo media and allowed to recover for three hours at 28.5°C before behavior testing. Lateral line ablation was confirmed by labeling the hair cells with the vital dye FM 4-64 (Thermo Fisher Scientific, Waltham, MA). Larvae (n=4) from each of the four groups were incubated in 0.5X E2 media containing 3 mM FM4-64 for 30 seconds followed by three rinses in embryo media. The larvae were anesthetized as stated above and imaged for red fluorescence (excitation/emission maxima ~515/640 nm) using a Leica dissecting

microscope at 80x magnification to assess lateral line function. Startle response was assessed in the remaining fish as outlined above.

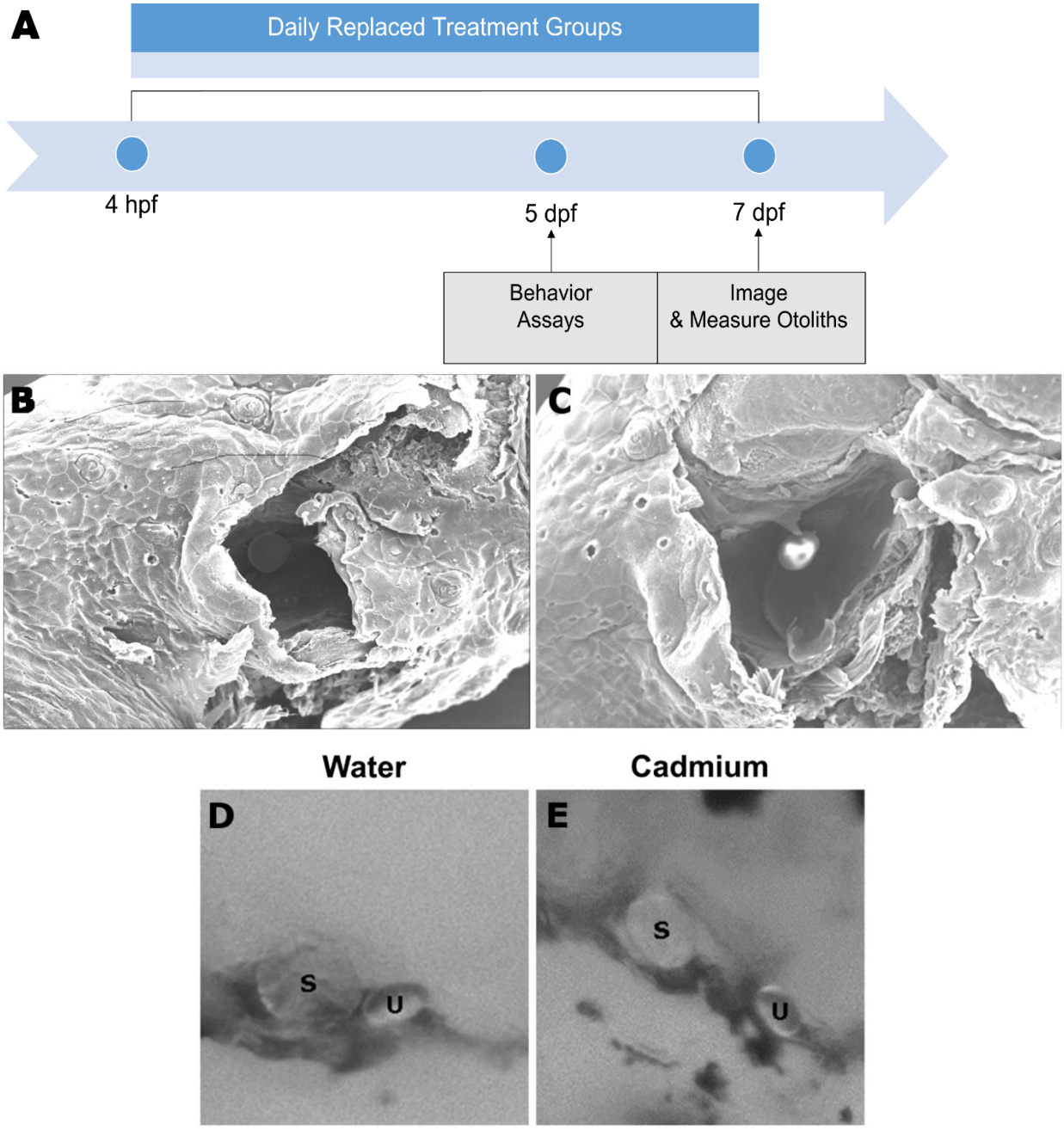

Figure 2: Study design for developmental Cd-exposure and its effects on otolith development. (A) Experimental timeline. *In situ* SEM images of the saccule otolith in the inner ear of (B) control and (C) Cd-exposed larvae. Bright field microscopy images of (D) control or (E) Cd-exposed zebrafish saccule and utricle otoliths. S – saccule, U – utricle

Radioassay to assess Cd uptake by larval zebrafish

To assess total body concentrations of Cd in zebrafish, triplicate groups of zebrafish embryos (n=50/group) were exposed from four hpf to seven dpf to 40 ppb Cd in the form of $CdCl_2$ in 0.5x E2, spiked with ^{109}Cd as a tracer (1592 Bq μg^{-1}) with or without 2.5 mM $CaCl_2$. Solutions were replaced daily during the course of the experiment. Larval uptake of Cd was monitored daily beginning at three dpf by measuring radioactive decay corrected for background activity. Briefly, larvae were washed three times with five ml of Cd-free 0.5x E2 media followed by transfer to clean scintillation vials in two mL of the final wash. An additional two mL of the final wash were transferred to a second clean scintillation vial to measure background activity. The radioactivity uptake was measured using a Wallac Wizard 1480 Gamma counter.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8.1.0)[41]. All data were analyzed for normality before statistical analysis using both the D'Agostino & Pearson normality test[44] and the Shapiro-Wilk normality test[45]. Non-normally distributed data were analyzed with the nonparametric Kruskal-Wallis test. The significance of the otolith size and calcium ion channel pharmacology was determined using a one-way ANOVA followed by the Holm-Sidak multiple comparison test was used as it is the most appropriate test for comparing a set of means[46]. This multiple comparison test was chosen as it has more power but cannot compute confidence intervals for the differences between means. Differences in otolith growth were determined using a repeated measures two-way ANOVA followed by Sidak's multiple comparisons test[47]. Rotational behavior during light/dark cycling was analyzed using a two-way ANOVA followed by Sidak's multiple comparisons test[48]. Rotational behavior with rescue

treatments was analyzed for main effects using a Kruskal-Wallis test followed by Dunn's multiple comparison test[49]. Relative otolith diameter with rescue treatments was analyzed using an ordinary one-way ANOVA followed by Tukey's multiple comparison test[48] as every mean was compared with every other mean. Startle sensitivity was analyzed for main effects using a Kruskal-Wallis test followed by Dunn's multiple comparison test[49].

Results

Cadmium induces changes in otolith diameter by inhibiting growth

Developmental Cd exposure resulted in a significant decrease in the size of the saccule otolith compared to unexposed controls as seen using both SE and bright field microscopy (Fig. 2). Using bright field images, we show that Cd exposure resulted in a concentration-dependent decrease in the diameter of the saccule otolith at seven dpf relative to untreated controls. The saccule otolith was markedly decreased in size beginning at 20 ppb Cd ($p<0.01$) with a maximal decrease observed at 40 ppb Cd treated ($p<0.001$) larvae compared to unexposed controls (Fig. 3a). The decreased otolith diameter leveled off at 40 ppb Cd and at higher concentrations, signs of gross toxicity were observed, including edema and lack of swim bladder inflation. Daily measurements of the saccule otolith beginning at one dpf showed that 40 ppb Cd did not significantly affect otolith nucleation (Fig. 3b) but did have a significant impact on the growth of the saccule otolith beginning at three dpf ($p<0.001$).

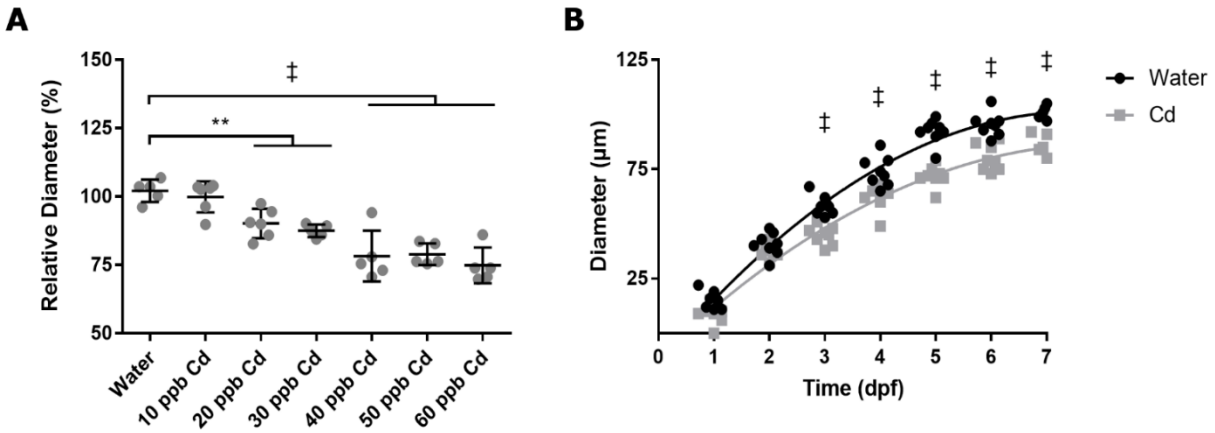

Figure 3: Saccule otolith size decreases in response to Cd-exposure. (A) Zebrafish saccule otolith size response to an exposure of 0 – 60 parts ppb Cd. (B) Diameter of zebrafish saccule otolith from one to seven dpf. when exposed to 40 ppb Cd. **p < 0.01, ‡p < 0.0001

These results demonstrate that developmental exposure to Cd, at concentrations comparable to human exposures[50], significantly inhibits the growth of the saccule otolith in larval zebrafish.

Cadmium induces behavioral changes associated with vestibular defects

To determine whether the otolith phenotype observed in Cd-exposed zebrafish correlated with changes in behavior, we assessed several behavioral endpoints. Exposure to 40 ppb Cd caused a significant increase in the number of rotations ($p<0.05$) observed during periods of light stimulus consistent with a defect in vestibular function (Fig. 4a). This circling behavior was characterized by a rapid twirling or spinning behavior in response to both mechanical and light stimuli and has been observed in other model organisms harboring mutations that affect the vestibular system[51,52]. Cadmium has been shown to induce oxidative stress[53] and interfere with divalent ion channels, in particular zinc and both voltage and non-voltage gated calcium channels[54,55]; therefore, we hypothesized that changes in otolith size and circling behavior were due to a Cd-mediated increase in reactive oxygen species (ROS) or its effects on calcium channels. To test this hypothesis, we co-exposed larvae to Cd and NAC (a ROS scavenger) or increasing levels of calcium. The inclusion of calcium in the exposure media was associated with a decrease in rotational behavior compared with Cd exposure alone ($p=0.973$) (Fig. 4b). In addition, we observed that increasing the amount of calcium in the exposure media rescued the decrease in saccule otolith size caused by Cd compared to untreated controls ($p>0.999$) (Fig. 4c). While NAC was unable to rescue the rotational behavior ($p<0.05$), it did result in a slight increase in the saccule otolith size compared to otoliths from Cd-only exposed zebrafish ($p<0.05$) but was still significantly decreased compared to controls ($p<0.0001$, Fig. 4b and c). Addition of

zinc to the exposure media did not rescue the Cd-induced decrease in the size of the saccule otolith ($p<0.001$) (Fig. 4d).

These results provide evidence that Cd-induced rotational behaviors are dependent on otolith malformations and vestibular defects by interfering with Ca homeostasis.

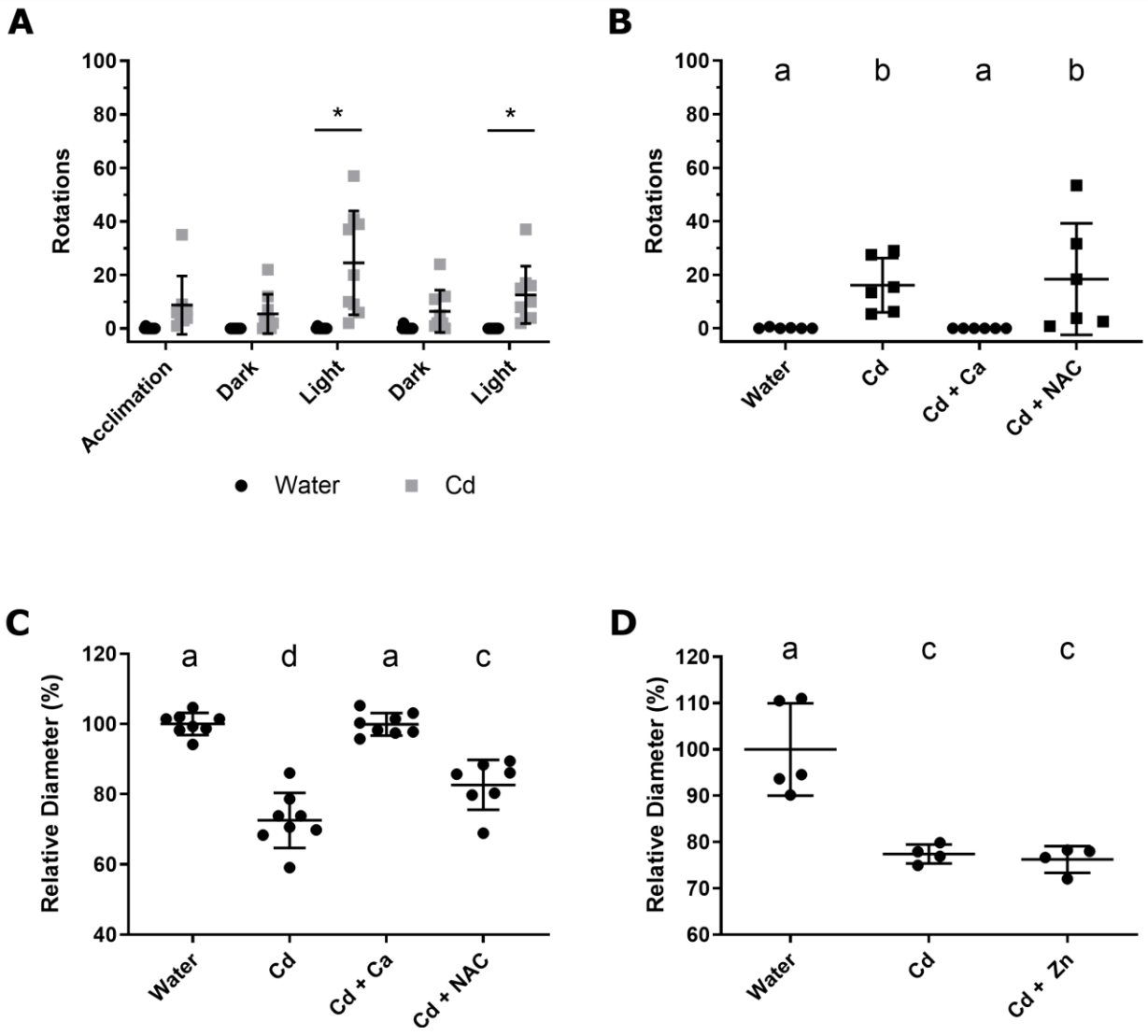

Figure 4: Otolith size dependent rotational phenotype. (A) Number of rotations in response to light and dark cycling. (B) Average number of rotations per well (n=8) in response to treatment. (C, D) Relative diameter of zebrafish saccule otolith at seven dpf. a-not significant, *$p < 0.05$, b-$p < 0.05$, c-$p < 0.0001$, d-$p < 0.0001$

Cd uptake by larval zebrafish

As shown in figure 5, once the embryos hatch at three dpf they begin to rapidly take up Cd from the media before exposure ended on seven dpf. Post-exposure, the larvae began to slowly eliminate Cd. Addition of Ca to the exposure media significantly reduced Cd uptake up by the larvae beginning at 4 dpf. These data show that Cd is readily taken up from the media by the larval zebrafish and that the addition of Ca to the exposure media results in a significant reduction in the amount of Cd incorporated into the larvae.

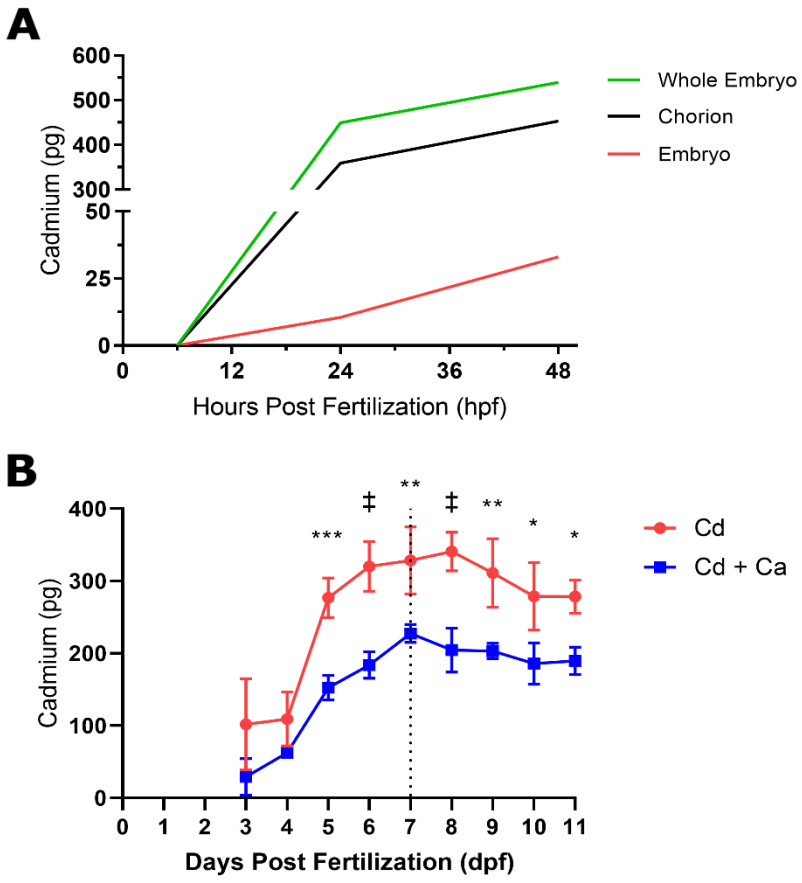

Figure 5: Cd uptake. (A) ^{109}Cd radio tracer uptake by zebrafish embryos from 4 hpf. (B) ^{109}Cd radio tracer uptake with rescue treatment after hatching, data indicated are means ± counting error. *p < 0.05, ** p < 0.01, *** p < 0.001, ‡p < 0.0001

Cadmium increases vestibular sensitivity to auditory stimuli

Acoustic startle responses are a reliable way to assess the function of the auditory portion of the vestibular system by five dpf [56]. As described by Burgess and Granato[37] the acoustic startle response can be divided into two distinct patterns of response, a short latency C-startle (SLC) and a long latency C-startle (LLC). The former is characterized by a rapid response (latency <17ms) to sound stimuli while the latter is any C-startle that occurs after 17 ms.

In this experiment zebrafish larvae were exposed to Cd and their C-startle frequency in response to increasing sound intensities was assessed. We observed a leftward shift in the SLC startle frequency curve (Fig. 6a), indicating that the larvae startle more frequently to lower intensity stimuli in the Cd-exposed group versus unexposed controls and that this response was concentration-dependent (Fig. 6b). Furthermore, this increase in acoustic sensitivity was the result of a shift in the type of C-startle response from LLCs to SLCs in the fish exposed to Cd (Fig. 6c). This hypersensitivity was quantified using the startle sensitivity index (Fig. 6b) showing that there was a significant increase in the number of SLCs ($p<0.05$) and a decrease in the number of LLCs ($p<0.0001$) in Cd-exposed larvae compared to controls. This Cd-induced hypersensitivity to auditory stimuli occurred independent of the lateral line (Fig. 6d) as ablation of the neuromasts with neomycin did not affect the Cd-induced sensitivity (Fig. s1). SEM images of the neuromasts indicated that Cd exposure from four hpf through seven dpf had no effect on the structure of the neuromast hair cells of the larval zebrafish (Figure 7). These data suggest that developmental Cd exposure results in vestibular dysfunction causing a significant hypersensitivity to auditory stimuli independent of the lateral line.

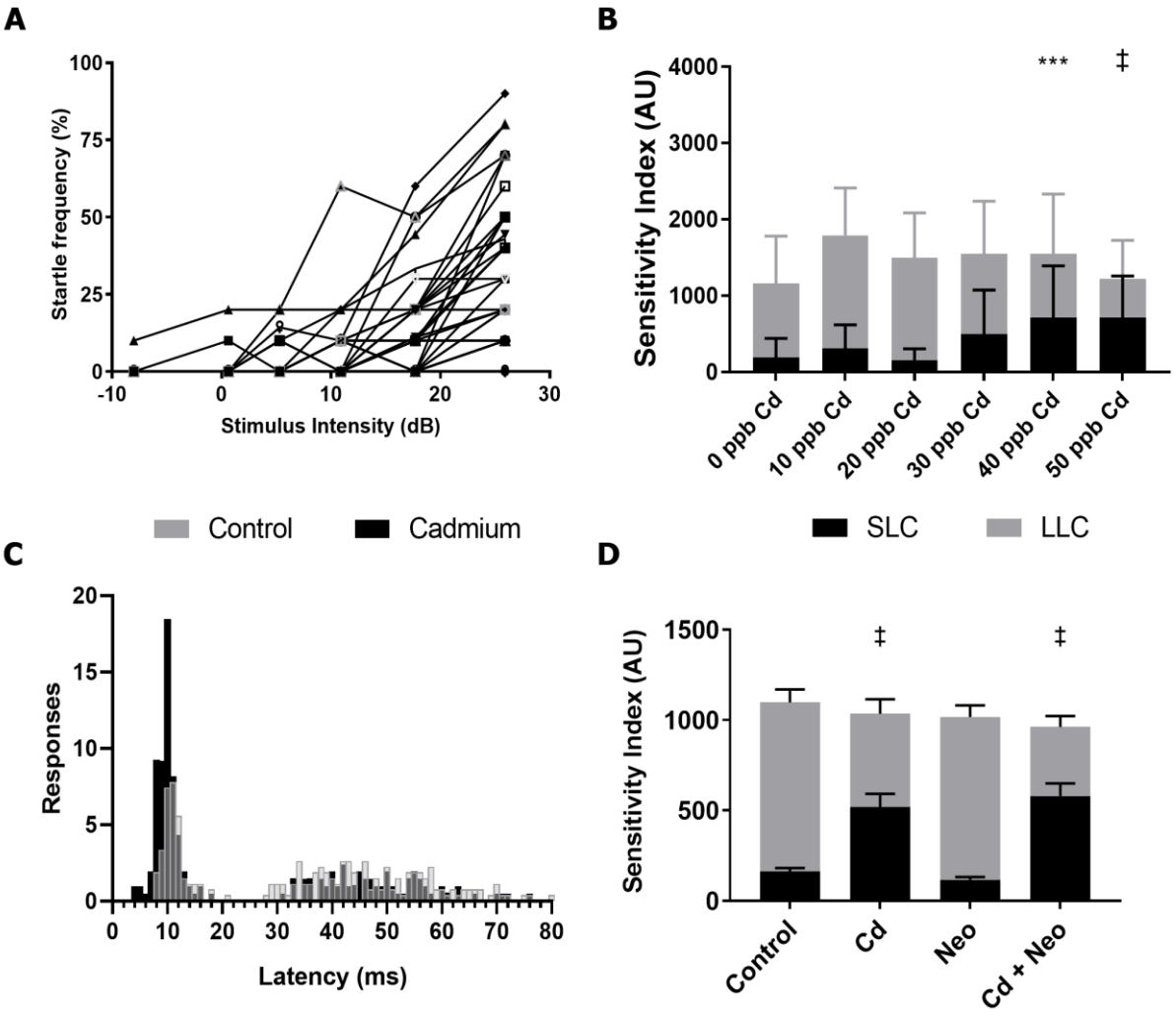

Figure 6: Cd-induced hypersensitivity to auditory stimuli. (A) Representative short latency c-startle (SLC) frequency curve from control larvae. (B) Startle sensitivity index for both the short latency (SLC) and long latency c-startle (LLC) cadmium concentration response. (C) Histogram of the latency to response for control vs 40 ppb Cd exposure. (D) Startle sensitivity index for both the short latency and long latency c-startle responses to 40 ppb Cd exposure with lateral line ablation by neomycin (Neo). *$p < 0.05$, *** $p < 0.001$, ‡$p < 0.0001$

Cadmium disrupts otolith formation through ligand gated calcium channels

Cadmium is known to interfere with the function of ion channels, particularly calcium ion channels[54,55]. We investigated two types of calcium ion channels: one found in the epithelial cells of the inner ear, intestines, and kidneys (Trpv6); and a member of a family of purinergic ion channels associated with otolith formation (P2rx).

Using pharmacological interventions, we showed that the Trpv6 antagonist, 2-APB, ($p=0.728$) and the P2rx agonist, Bz-ATP, ($p=0.631$) were able to rescue the Cd-induced decrease in otolith size (Fig. 8a and 8b). Exposure to the P2rx antagonist (NF023) decreased the size of the saccule otolith ($p<0.01$) and when combined with Cd resulted in a further decrease in otolith size ($p<0.0001$). While the role of P2rx in otolith development has not been completely characterized, there is evidence supporting a link between P2rx and the calcium regulatory gene *otop1*[62]. These data indicate that Cd-induced reductions in saccule otolith size are the result of interference with inner ear calcium levels through disruption of the P2rx calcium ion channel.

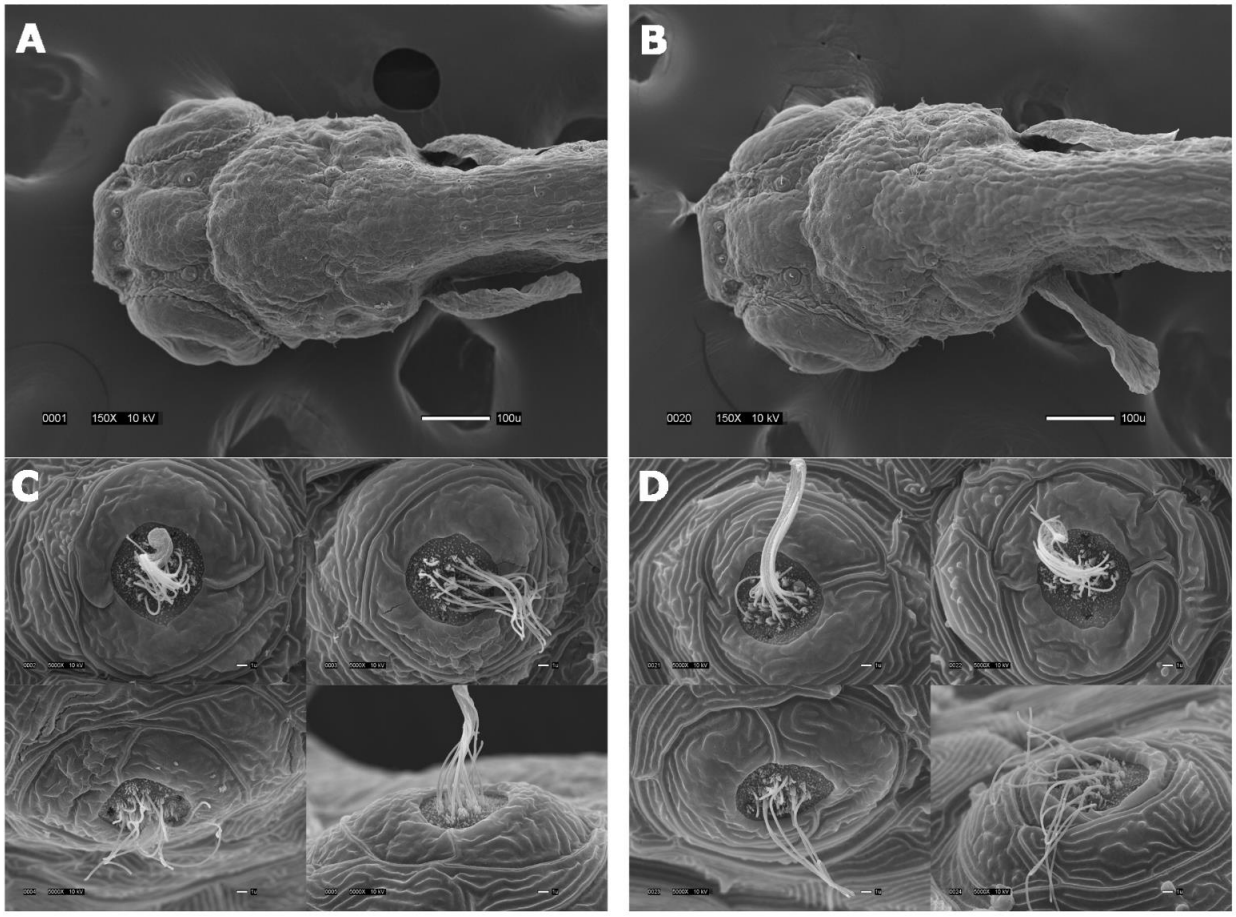

Figure 7: SEM imaging showing neuromast response to Cd exposure. (A) Representative SEM image of an unexposed zebrafish. (B) Representative SEM image of a zebrafish exposed 40 ppb Cd. (C) High magnification images of unexposed individual neuromast cells. (D) High magnification images of individual neuromast cells after exposure to 40 ppb Cd.

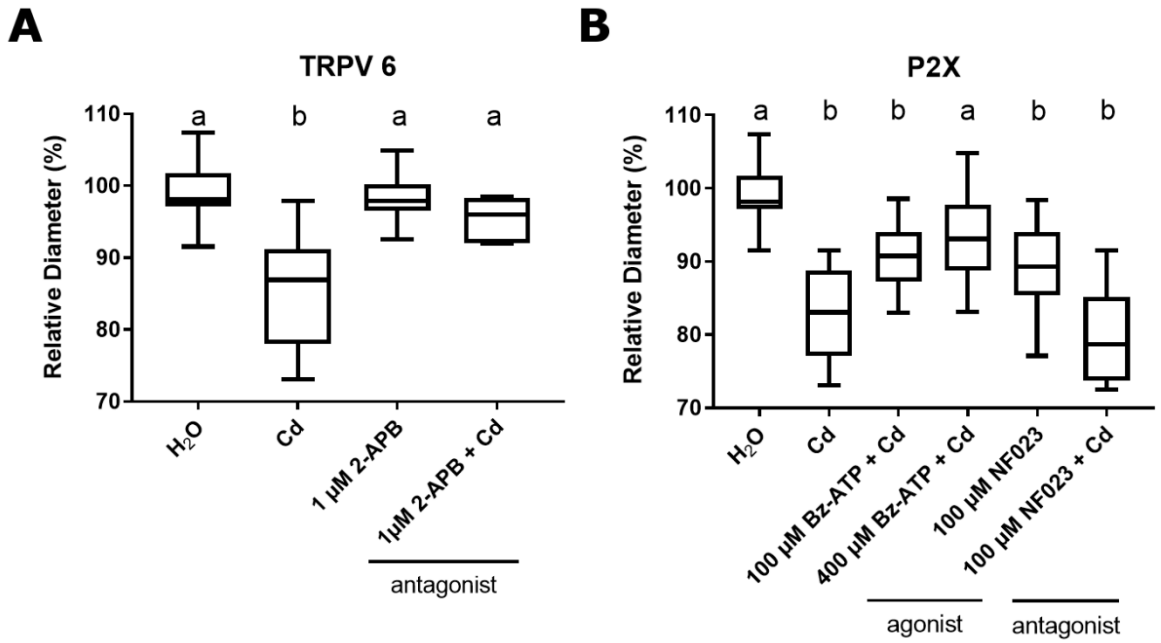

Figure 8: Disruption of calcium ion channels alters otolith size. (A) Changes in otolith size following co-exposure to Trpv6 antagonist. (B) Changes in otolith size following co-exposure to P2rx agonist and antagonist. a-not significant, b-$p < 0.05$

Discussion

Our results demonstrate that exposure to Cd during development significantly inhibits growth of the saccule otolith in larval zebrafish and provides evidence that Cd-induced rotational behaviors are dependent on otolith malformations. As shown in figure 3a, Cd exposure results in a concentration-dependent decrease in the size of the saccule otolith starting at 20 ppb Cd ($p < 0.01$) and continuing until 40 ppb Cd ($p < 0.0001$). Further increases in the Cd concentration above 40 ppb did not result in any additional decrease in otolith size. This observed effect of Cd exposure on otolith size was the result of Cd interfering with otolith growth ($p < 0.0001$) without having a significant effect on the nucleation of the otolith (Fig. 3b), though this needs to be investigated further. Our data show that this effect on otolith size results in circling behavior rarely observed in the unexposed controls and that this phenotypic response was more common during light versus dark periods (Fig. 4a). To show that Cd-induced decrease in otolith size was the cause of the observed increase in the number of rotations, zebrafish were co-exposed to Cd and calcium or NAC as Cd is known to interfere with calcium ion channels and induce ROS. We observed that the addition of calcium was able to rescue both the decrease in otolith size and circling while NAC had no significant effect on either phenotype. To our knowledge this is the first time that Cd has been shown to alter the vestibular system resulting in a significant effect on rotational behavioral. In addition, we show that this disruption of the vestibular system causes hypersensitivity to auditory stimuli. Exposure to Cd increased the sensitivity to sound stimuli as measured by the acoustic C-startle response in a concentration-dependent manner (Fig. 5b). We also show that this increase in sensitivity is the result in a shift of the type of startle response from LLCs to SLCs (Fig. 5c) beginning at 40 ppb Cd ($p < 0.001$). As neomycin ablation (Fig. s1) of the lateral line had no effect on Cd-induced increases in SLCs ($p < 0.0001$) and SEM images

show no discernable changes in the structure of the neuromast hair cells (Fig. 6), we can infer that the hypersensitivity observed is independent of the neuromast cells of the lateral line (Fig. 5d).

While the mechanism by which Cd disrupts otolith development needs to be further elucidated, our data indicate that the reduction in saccule otolith size may occur through interference of the function of P2X calcium ion channels. As shown in figure 7b, by treating Cd-exposed embryos with a general P2X agonist, Bz-ATP, we were able to rescue the Cd-induced decrease in otolith size. Conversely, treating embryos with NF023, a general P2X antagonist, we observed a significant decrease in otolith size ($p < 0.001$), which were decreased further with the addition of Cd ($p < 0.0001$) (Fig. 7b). In zebrafish there are seven P2X isoforms: P2rx1, P2rx2, P2rx3, P2rx4, P2rx5, P2rx7, and P2rx8. They are expressed in a range of tissues but only P2rx2, P2rx3, P2rx4, P2rx7, and P2rx8 appear to be expressed in the developing otic vesicle[57,58]. Cd is known to potentiate P2rx2 and P2rx4 but inhibit P2rx1, P2rx3, and P2rx7, whereas NF023 inhibits and Bz-ATP activates all P2X isoforms[59–61]. Therefore, it is reasonable to speculate that the vestibular disruption observed is mediated through either P2rx3 and/or P2rx7 as these are the only two subunits known to be expressed in the inner ear that are also inhibited by Cd. These data demonstrate that developmental exposure to Cd at concentrations comparable to those observed in human cohorts[50] results in significant structural defects with associated vestibular effects. The auditory structural defects induced by Cd are of particular concern as, unlike zebrafish in which otoliths continue to grow in size as the fish ages, the size and number of otoconia found within the inner ear of mammals changes very little after the early postnatal period[63,64]. Further studies are needed to determine cadmium's molecular target within the vestibular system and which development timepoints are the most susceptible to Cd ototoxicity.

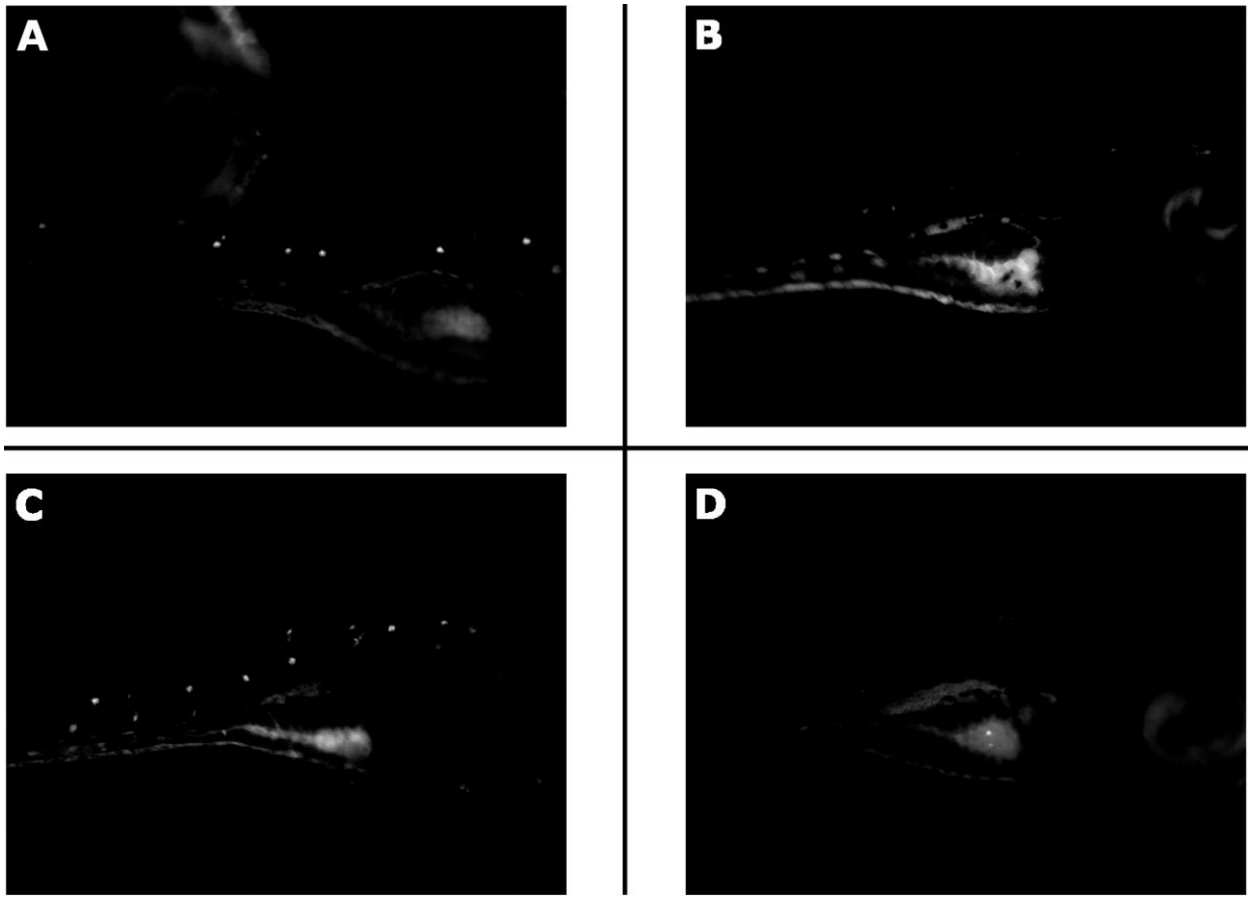

Figure s1: Neomycin ablation of the lateral line. Fluorescent images of FM 4-64 taken up by neuromast hair cells of the lateral line in 5 dpf zebrafish larvae. (A) Unexposed controls. (B) Controls exposed to 50 µM neomycin. (C) Larvae exposed to 40 ppb Cd. (D) Larvae exposed to 40 ppb Cd and 50 µM neomycin.

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CHAPTER 5

Conclusions and Future Directions

Conclusions

Although genetic predisposition and energy imbalance are risk factors for obesity in children, caloric excess and reduced physical activity alone do not fully account for the obesity epidemic[1]. There is modest evidence in the literature showing that Cd exposure in adult animals is associated with metabolic syndrome both *in vitro* and *in vivo*, but there are limited data evaluating the association between developmental exposure to Cd and obesity[2].

In chapter 2 we evaluated the association between prenatal Cd exposure and obesity in children, and determined the plausibility of this relationship in a controlled experimental zebrafish model. We found a persistent association between prenatal Cd exposure and increased risk of obesity from birth to age five years in the NEST cohort. The average first trimester blood Cd levels were 0.3 ng/g blood weight, which equates to 483 ng/g of body weight[3]. In support of this association, zebrafish exposed developmentally to Cd at 60 ppb from four hpf had an estimated Cd body burden at seven dpf of 386 ng/g of body weight. These fish went on to exhibit significantly higher lipid accumulation as juveniles, when compared to unexposed controls. Surprisingly, lipid accumulation and body weight plateaued in both the zebrafish model and in the human data, respectively. To our knowledge, this represents the first direct measure of an association between developmental Cd exposure and increased obesity risk in children, the results of which are supported by similar findings in zebrafish, an evolutionarily related model organism. To understand the mechanisms behind the changes in zebrafish lipid accumulation, and the association between prenatal Cd exposure and body weight, hADMSCs were exposed to Cd during adipogenic differentiation (Chapter 3).

We showed that hADMSCs exposed to 0.5 and 5 ppb Cd, during adipogenic differentiation, exhibited significant increases in the number of differentiated adipocytes while hADMSCs exposed to 5 ppb Cd showed increased lipid accumulation compared to unexposed controls. This exposure concentration is similar to that of Cd found in maternal and cord blood of people from developed countries, ranging from 0.03 to 9.17 ppb[4–11]. The placenta may moderately protect a developing fetus from exposure but epidemiological data highlight that fetal / newborn MSCs are likely exposed to Cd at concentrations very similar to those we have demonstrated have the ability to increase adipocyte differentiation and lipid accumulation[12–15]. Recent developmental and early life animal studies show that exposure to Cd increases total fat mass, circulating lipids, and bone marrow yellowing, and decreases bone volume[16].

The osteotoxicity effects of Cd on bone have been well established in the literature and include increased risk of bone fractures[17–19], osteoporosis[19–22], and skeletal deformities[23], along with decreased bone mineral density and expression of bone marker genes, including *spp1* and *sparc* [24–27]. Considering that adipocytes, chondrocytes, and osteocytes arise from MSCs we evaluated the effects of Cd exposure on osteogenesis in developing zebrafish embryos. We observed a Cd-induced decrease in bone mineralization in a concentration-dependent manner in the notochord, pharyngeal teeth, and cleithrum all of which exhibited a loss of mineralization at eight dpf in larvae exposed to 15 ppb Cd and higher. Furthermore, following 14 days of chronic exposure to Cd, we observed a noticeable decrease in larval bone mineralization beginning at 25 ppb, whereas lower concentrations were indistinguishable from controls. However, there was no disruption of chondrogenesis and all cartilaginous structures were present. These results suggest that Cd inhibits but does not abolish the mineralization process of bone development. The

consequences of such an inhibition could include weak and/or brittle bones; defects in bone growth, including elongation; and alterations in bone patterning.

In Chapter 4 we transitioned to investigate how cadmium induces changes in the vestibular system and how these changes result in aberrant behavioral responses. Cd is associated with balance and vestibular dysfunction in adults exposed to Cd occupationally, but little is known about the developmental effects of low concentration Cd exposure. We demonstrated that exposure to Cd during development inhibits the growth of the saccule otolith in larval zebrafish and offer evidence that Cd-induced rotational behaviors are dependent on otolith malformations. Cd exposure results in a concentration-dependent decrease in the size of the saccule otolith starting at 20 ppb while Cd concentrations above 40 ppb did not result in any additional decrease in otolith size. We speculate that this Cd-induced decrease in otolith size is the result of Cd interfering with otolith growth via mineralization without disturbing the nucleation of otolith precursor proteins. Our data show that this effect on otolith size results in circling behavior rarely observed in the unexposed controls, and hypersensitivity to auditory stimuli as measured by the acoustic C-startle response. This increase in sensitivity is the result in a shift of the type of startle response from LLCs to SLCs independent of the neuromast cells of the lateral line. While the mechanism by which Cd disrupts otolith development needs to be further elucidated, the reduction in saccule otolith size may occur through interference of the function of P2X calcium ion channels. In favor of the former, treatment of Cd-exposed embryos with a general P2X agonist, Bz-ATP, rescued the Cd-induced decrease in otolith size. Conversely, treating embryos with NF023, a general P2X antagonist, resulted in a significant decrease in otolith size, which were decreased further by the addition of Cd.

Proposed mechanisms of Cd adipogenic, osteogenic, and vestibular disruption

Calcium (Ca) is involved in many cellular functions including differentiation, proliferation, apoptosis, and cell death. It has been reported that Ca has potentially a dual effect on adipogenesis by inhibiting the early stages and stimulating the later stages[28–30]. A study by Goudarzi et al. showed that by increasing intracellular Ca levels, adipogenesis could be restrained in hADMSCs[31]. If we next consider that vitamin D plays a vital role in maintaining Ca homeostasis and that vitamin D increases osteogenesis and decreases adipogenesis in MSCs, it is reasonable to hypothesize that Cd disrupts Ca homeostasis through ion channels activated by vitamin D[26,32]. MSCs express L-type calcium channels whose activity is suppressed by the addition of Cd and increased by vitamin D receptor agonists[33,34]. Therefore, one potential mechanism by which Cd exposure increases the differentiation of adipocytes and increases lipid accumulation is through a decrease in intracellular Ca concentration by interfering with the activity of L-type calcium channels (*Cacna1c*).

The data presented in chapter 3 shows that Cd decreases bone mineralization. While Ca plays a pivotal role in osteogenesis and bone mineralization, the literature suggests that Cd disrupts osteogenesis through induction of ROS rather than through interference with cellular Ca homeostasis[35]. Our data shows that ROS does not account for all the pleiotropic effects of Cd. In particular, we show that both rotational behavior and otolith development are through a ROS-independent Ca-dependent mechanism. Therefore, it is reasonable to question how much of our observed decrease in mineralization is through ROS induction or the result of Cd interference of Ca homeostasis.

Similar to the suggested mechanism for the Cd-induced increase in adipogenesis, the data presented in chapter 4 show that disruption of vestibular structure and function is driven through

endolymph Ca homeostasis. Pharmacological intervention using Bz-ATP and NF023 implicates the P2x calcium ion channel. In zebrafish, there are seven P2X subunits P2rx1 - P2rx5 along with P2rx7, and P2rx8. They are expressed in a range of tissues, including smooth and cardiac muscles; the central and peripheral nervous system; retina; as well as epithelial, endothelial, renal, and pituitary cells, but only P2rx2, P2rx3, P2rx4, P2rx7, and P2rx8 appear to be expressed in the developing otic vesicle[36,37]. Considering that Cd is known to potentiate P2rx2 and P2rx4, but inhibit P2rx1, P2rx3, and P2rx7, and that NF023 inhibits while Bz-ATP activates all P2X subunits[38–40], it is reasonable to suspect that the vestibular disruption observed is mediated through P2rx3 and/or P2rx7.

Based on studies and data presented here, we propose that the Cd-induced decrease in otolith size is the result of one or a combination of the following: Direct interference with or incorporation of Cd into the calcium carbonate crystal thereby disrupting the formation of the otolith crystal matrix; indirectly by competitively inhibiting the P2rx3 and/or P2rx7 calcium ion channels. The indirect effect would result in increased levels of calcium in the endolymph affecting scaffold protein binding to the otoliths. Inhibition of the Trpv6 ion channel with 2-APB did not decrease the size of the saccule otolith. While Trpv6 is expressed in the inner ear it is also highly expressed in the gills of zebrafish and plays a major role in calcium uptake. Therefore, inhibition of Trpv6 likely reduced Cd uptake and reduces its disruptive effects. A similar response can be seen with addition of Ca to the media. This results in a rescue of the otolith and the ^{109}Cd uptake data presented in chapter 4 shows a decrease in Cd uptake when supplemental

Ca is added to the exposure media.

The data presented here demonstrates that Cd disrupts otolith formation by interfering with Ca homeostasis through one of two possible mechanisms (Fig 1). The first is that Cd disrupts calcium carbonate crystal formation either by directly incorporating into the otolith crystal matrix or by interfering with calcium carbonate ion deposition into the matrix. Second, is potentially through inhibition of P2rx3 or P2rx7 ion channels by Cd resulting in a buildup of Ca ions in the normally Ca poor endolymph affecting scaffold protein binding to the growing otolith.

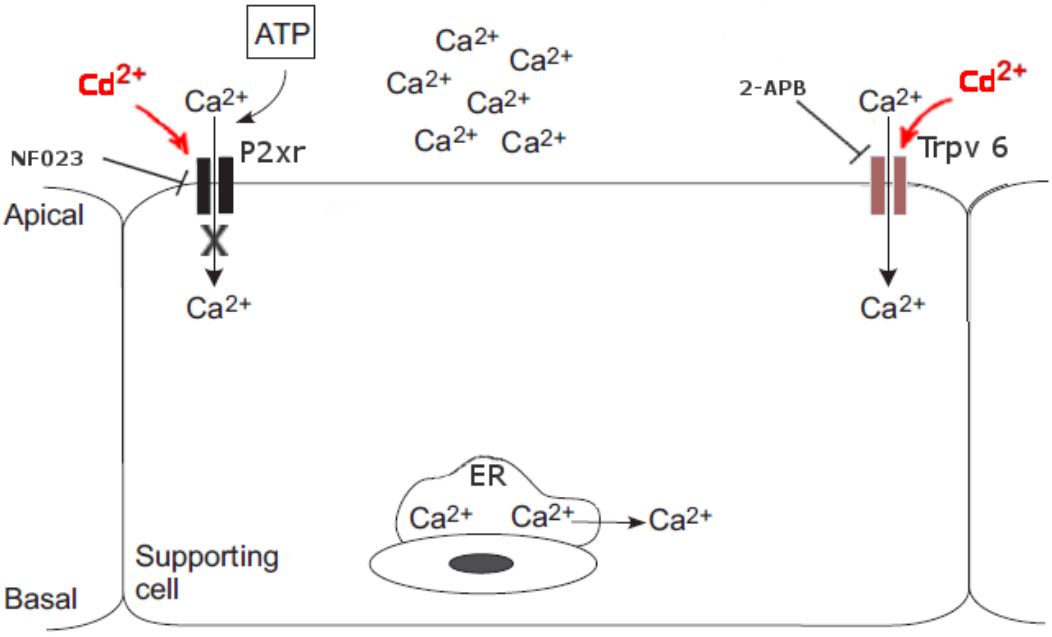

Figure 1: Proposed mechanism of otolith disruption by Cd. Adapted from Kim et al. (2010). Cd inhibition of otolith growth is hypothesized to be the result of (1) disruption of calcium carbonate crystal formation through interference or incorporation of Cd into the otolith crystal matrix, (2) competitive inhibition of P2rx3 or P2rx7 by Cd resulting in high levels of calcium ions in the endolymph affecting scaffold proteins binding to the otoliths.

Future directions

In chapters 2 and 3 we have shown that developmental Cd exposure can increase adipogenesis both *in vivo* and *in vitro* but the underlying mechanisms still needs to be elucidated. This could be achieved by conducting gene expression analysis of adipogenic transcription factors in hADMSCs following Cd exposure and evaluating the effects of Cd exposure on MSC calcium homeostasis. Secondly, in chapter 3 we have shown that Cd exposure delays osteogenesis *in vivo* but to determine whether this is caused by a decrease in osteocyte differentiation needs to be assessed by analyzing the gene expression of osteogenic gene markers such as *runx2, spp1, col1a1, sp7, sparc,* and *tnfs11*. Additionally, it is unclear whether Cd exposure results in niche specific effects. Therefore, the effects of Cd exposure on adipogenesis and osteogenesis in hBDMSCs needs to be assessed. Finally, future studies should be conducted in MSC's derived from both males and females as animal data suggests that there is increased susceptibility in males.

Figure 1 shows that Cd disrupts vestibular development through one of two possible mechanisms. To determine the contribution of each to the observed phenotype, subtype-specific agonists and antagonist could be used to determine which of the seven P2xr channels is the molecular target of Cd within the vestibular system. Secondly, the changes in vestibular calcium could be evaluated using ratiometric calcium indicators along with assessment of the otoliths using inductively coupled plasma mass spectrometry to determine Ca and Cd incorporation.

Considering the ubiquitous and persistent nature of environmental Cd and the risk of exposure, the results presented along with the experiments proposed above will help elucidate the mechanism of Cd exposure and thereby aid both regulators and public health officials reduce the negative impacts of developmental Cd exposure.

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APPENDIX

APPENDIX A

Developmental Cadmium Exposure Induces ROS Dependent Hyperactivity in Larval Zebrafish

Abstract

The present study was conducted to determine the effect of Cadmium (Cd) exposure on neurobehavioral development and oxidative stress by exploring whether Cd is capable of inducing behavioral changes in larval zebrafish in a concentration-dependent, mediated by reactive oxygen species (ROS). One mechanism of Cd toxicity is through the production of ROS. While Cd is unable to generate free radicals directly, it facilitates the production of ROS through depletion of the ROS scavenger molecule glutathione (GSH), mitochondrial damage, induction of NADPH oxidases, and replacement of redox-active elements such as iron[1]. ROS have been shown to negatively impact the developing nervous system resulting in cognitive impairment in animal models. Zebrafish embryos were exposed to 0 to 60 ppb Cd from four hpf through five dpf and larval behavioral response to dark-light cycling was assessed at five dpf. We observed that Cd exposure resulted in a threshold-dependent increase in distance moved during dark periods compared to unexposed controls at 30 ppb Cd. To elucidate the mechanism, the ROS scavengers N-acetylcysteine (NAC) and Vitamin C (AA) were used to assess the role of ROS in Cd-induced behavioral changes. Co-exposure to 40 ppb Cd and either 1 mg/L NAC, or 5 µg/mL AA was able to rescue the Cd-induced changes in distance moved in the dark observed in Cd exposure alone. Considering that Cd causes mitochondrial damage or directly interacts with GSH resulting in increased hydrogen peroxide (H_2O_2) and free radical production, and NAC aids in

GSH synthesis while AA neutralizes free radicals directly, our results suggest that the major drivers of Cd-induced hyperactivity are free radical or H_2O_2 mediated.

Introduction

Neurodevelopmental disorders (NDD) are extensive and complex and can be caused by genetic or environmental factors. Unlike other organs that are formed at specific times during development, the nervous system develops throughout all stages including adulthood, but it is particularly vulnerable to chemical exposure early in life. Therefore, the effects of neurotoxic substances can produce very different phenotypes depending on the timing of exposure. These can include changes to neurogenesis, cell proliferation, cell migration, or natural apoptotic processes, which could lead to harmful effects on the morphology of the developing nervous system. This can result in intellectual disabilities, communication disorders, learning disorders, autism spectrum disorders, epilepsies, and motor and coordination disorders.

Cd is a ubiquitous environmental pollutant that has been shown to cause behavioral and cognitive changes in human and animal studies. Perinatal exposure to Cd in rodents causes a delay in sensorimotor development[2], increased spontaneous motor activity[3], and decreases in learning ability of offspring[4]. The neurological effects of Cd have been examined in a variety of fish species. Results of these studies suggest that Cd is capable of increasing auditory thresholds, impairing social and escape behavior, damaging the sensory macula, and increasing antioxidant and detoxifying gene expression [5–10]. While in human populations, studies have assessed the neurodevelopment of young children after low-level cadmium exposure during pregnancy, these studies have reported mixed results though developmental delay is common[11–13].

The molecular mechanism by which Cd induces damage is still under investigation though studies have shown that Cd depletes protein-bound sulfhydryls and GSH, causes lipid peroxidation, alters DNA structure and the activity of antioxidant enzymes, and changes the structure and function of cell membranes, all of which can result from oxidative stress and

oxidative tissue damage[14–16]. Oxidative stress is the result of overproduction of ROS and/or disturbances of the oxidant defense system[17]. The oxidant defense system comprises antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), the selenium-independent glutathione-S-transferase (GST) and selenium-dependent glutathione peroxidase (GPX), and non-enzymatic antioxidants such as vitamins C and E, carotenoids, thiol antioxidants, and natural flavonoids[14,18].

Zebrafish display many complex behavioral patterns, which are highly comparable to rodents and humans, and is an excellent model organism to study developmental chemical exposure and ROS[7,9,10]. Two recent studies using zebrafish looked at how developmental exposure to Cd affected behavior in larvae and adult fish. The first exposed zebrafish embryos to 1 µM (112 ppb) Cd and found increased *sod, cat,* and *gpx* gene expression along with increased SOD and decreased GPX activity levels[10]. The second study showed that 1 µM Cd induced an increase in average movement in adults and decreased GPX and GSH following acute embryonic exposure[9]. These indicate that developmental Cd exposure is capable of inducing behavioral changes at or above the upper limit of human exposure and that exposure results in disturbance of the oxidant defense system. However, considering that Cd can influence a multitude of cellular processes, the link between Cd-induced behavioral changes and ROS still needs to be elucidated[1,19]. In the present study, we examined how developmental Cd exposure affects larval zebrafish behavioral response to light and dark stimuli and how these responses are altered by antioxidant treatment.

Materials and Methods

Animal husbandry

Wildtype (AB strain) zebrafish were maintained in the NC State University Zebrafish Core Facility according to standard protocols. All work involving zebrafish was approved by the NC State Animal Care and Use Committee.

Chemicals

A stock solution of cadmium chloride (99.99% purity; Sigma-Aldrich, St Louis, MO) was dissolved in reagent-grade (Picopure®) water at 10 ppt and stored at -20˚C in 1.5 mL polypropylene tubes. 10 - 60 ppm (1000x) substocks were made and stored at room temperature in 1.5 mL polypropylene tubes. N-Acetyl Cysteine ([NAC], >99% purity; Sigma-Aldrich, St Louis, MO) was dissolved in reagent-grade water at 25 mg/mL and stored at room temperature. Ascorbic Acid ([AA], >99% purity, Sigma-Aldrich, St Louis, MO) was dissolved in reagent-grade water at 5 mg/mL and stored at -20˚C in 1.5 mL polypropylene tubes.

Exposures

Zebrafish embryos were collected immediately after spawning and exposed to 10 to 60 ppb Cd in 0.5X E2 media (7.5 mM NaCl; 250 µM KCl; 500 µM $MgSO_4$; 75 µM KH_2PO_4; 25 µM Na_2HPO_4; 500 µM $CaCl_2$; 350 µM $NaHCO_3$) from four hpf through five dpf at a density of 10 embryos/mL. This concentration range was selected as it represents the upper range observed in human populations exposed to cadmium-polluted environments. The media was replaced daily and feeding began at five dpf. We co-exposed zebrafish to 40 ppb Cd and 1000 µg/L NAC, or 5 µg/mL AA after puncturing the chorion with a 1µm needle prior to exposure.

Radioassay to assess Cd uptake by larval zebrafish

To assess total body concentrations of Cd in zebrafish, triplicate groups of zebrafish embryos (n=50/group) were exposed from four hpf to seven dpf to 40 ppb of Cd in the form of $CdCl_2$ in 0.5x E2, spiked with ^{109}Cd as a tracer (1592 Bq μg^{-1}). Solutions were replaced daily during the course of the experiment. Larval uptake of Cd was monitored daily beginning at three dpf by measuring radioactive decay corrected for background activity. Briefly, larvae were washed three times with five ml of Cd-free, non-radioactive 0.5x E2 media followed by transfer to clean scintillation vials in two mL of the final wash. An additional two mL of the final wash were transferred to a second clean scintillation vial to measure background activity. The radioactivity uptake was measured using a Wallac Wizard 1480 Gamma counter. As the chorion could potentially act as a barrier to Cd uptake a subset of embryos were measured and then dechorionated. Following dechorionation the larvae and chorions were assessed for radioactivity separately.

Behavior assays

Behavior was assessed using a DanioVision^{TM} box with EthoVision® XT software (Noldus; Leesburg, VA). Behavior for all exposures was conducted at five dpf. Zebrafish larvae were arrayed in a 96-well plate with 200 µL of media in each well and Plates were placed in the DanioVision^{TM} box after which the larvae were allowed to acclimate in the dark for at least 30 minutes. Following acclimation, larval response to three 10-minute dark-light cycles was measured. Measured end points included movement, acceleration, and velocity. As zebrafish

larvae are more active in the dark than in the light[20], cumulative distance moved in the dark was used to compare treated vs. controls.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8.1.0)[21]. All data was analyzed for outliers using the ROUT test with a 2% Q value[22], and normality using both the D'Agostino & Pearson normality test[23] and the Shapiro-Wilk normality test[24] before statistical analysis. If the data were not normally distributed, the nonparametric Kruskal-Wallis test was used; otherwise an ordinary one-way ANOVA was used followed by the Holm-Sidak multiple comparison test[25].

Results

Cadmium induces hyperactive-like behavioral changes in larval zebrafish

Developmental exposure to Cd causes an increase in distance moved during dark periods consistent with a hyperactive-like phenotype. As seen in Figure 1, zebrafish larvae display characteristic activity patterns in response to light-dark cycling[20]. This pattern is characterized by an initial burst of activity that subsides with continuing darkness. This normal pattern of activity is altered by exposure to 40 ppb Cd. Larvae (n=48) exposed to Cd exhibited a heightened level of activity throughout the majority of the behavioral assay, but these differences were most pronounced during dark periods (Fig 1). Integrating the area under the curve over multiple dark periods (Fig 1 insert) results in a significant increase in distance moved in Cd-exposed fish ($p < 0.0001$). These data show that Cd-induces a significant increase in distance moved in response to light-dark cycling in 5 dpf larval zebrafish.

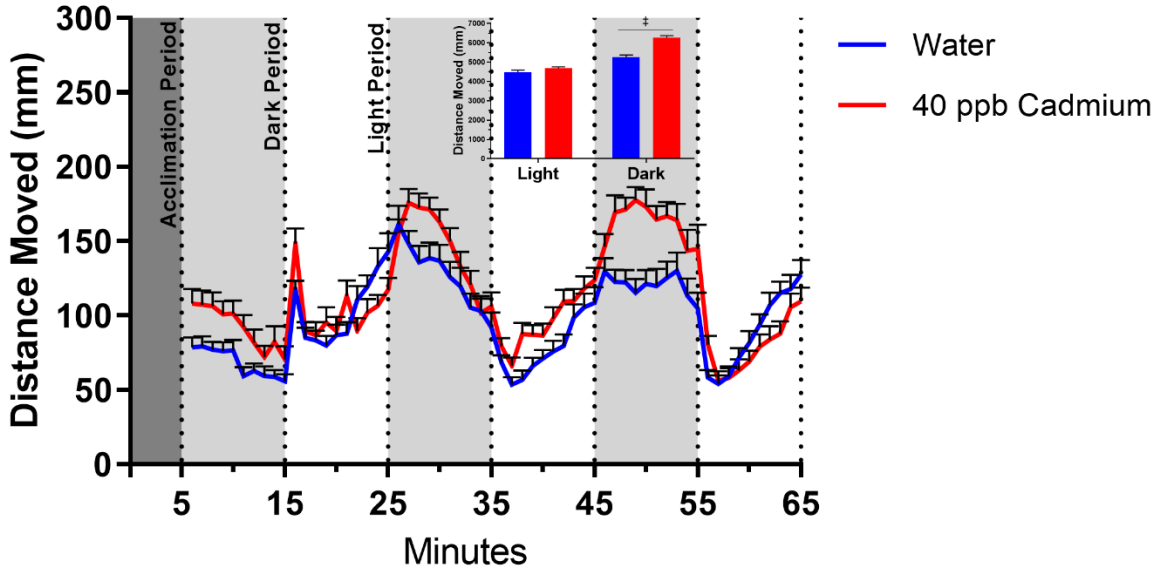

Figure 1: Cd induces hyperactivity during light-dark cycling in zebrafish larvae at five dpf. Insert shows area under the curve for light and dark periods. ‡ $p < 0.0001$

Cd-Induced hyperactivity is threshold dependent

Developmental exposure to Cd causes a threshold dependent increase in distance moved during the dark periods. Zebrafish larvae exposed to 0 to 60 ppb Cd from four hpf through five dpf (Fig 2) display significant increases in distance moved during dark periods beginning at 30 ppb Cd ($p < 0.01$). Once this threshold concertation was reached, further increases in the Cd concentration did not result in further increases in distance moved. Larvae exposed to 60 ppb Cd began to display signs of toxicity at five dpf including edema and lack of swim bladder inflation. These data show that Cd induces a significant threshold-dependent increase in distance moved in response to light-dark cycling in five dpf larval zebrafish.

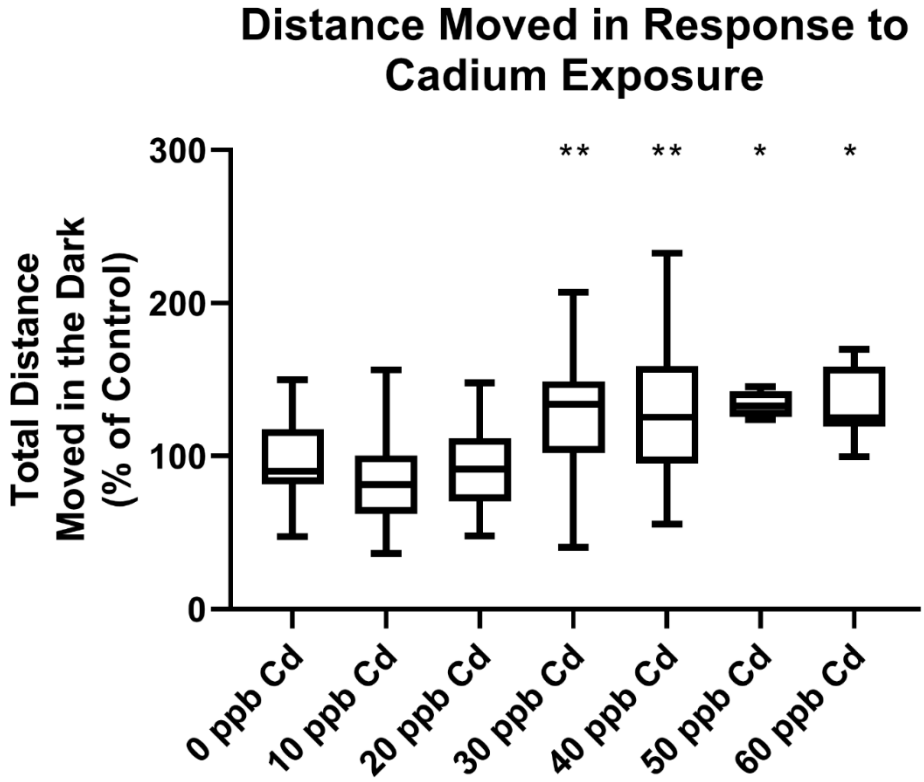

Figure 2: Total distance moved in the dark in Cd-exposed zebrafish larvae at five dpf.
* $p < 0.05$, ** $p < 0.01$

Cd uptake by larval zebrafish

The chorion acts as an effective barrier against Cd uptake into larval zebrafish. The data shown in figure 3a shows that the majority of the ^{109}Cd activity observed in the whole embryo is due to ^{109}Cd sequestering on the chorion during the first 48 hpf. Once the embryos hatch at three dpf they begin to rapidly take up Cd from the media before exposure end on seven dpf (Fig. 3b). After exposure was ceased, the larvae began to slowly eliminate Cd. These data show that while larvae take up Cd from the media during early development the chorion does act as a significant barrier protecting the larvae from environmental exposures.

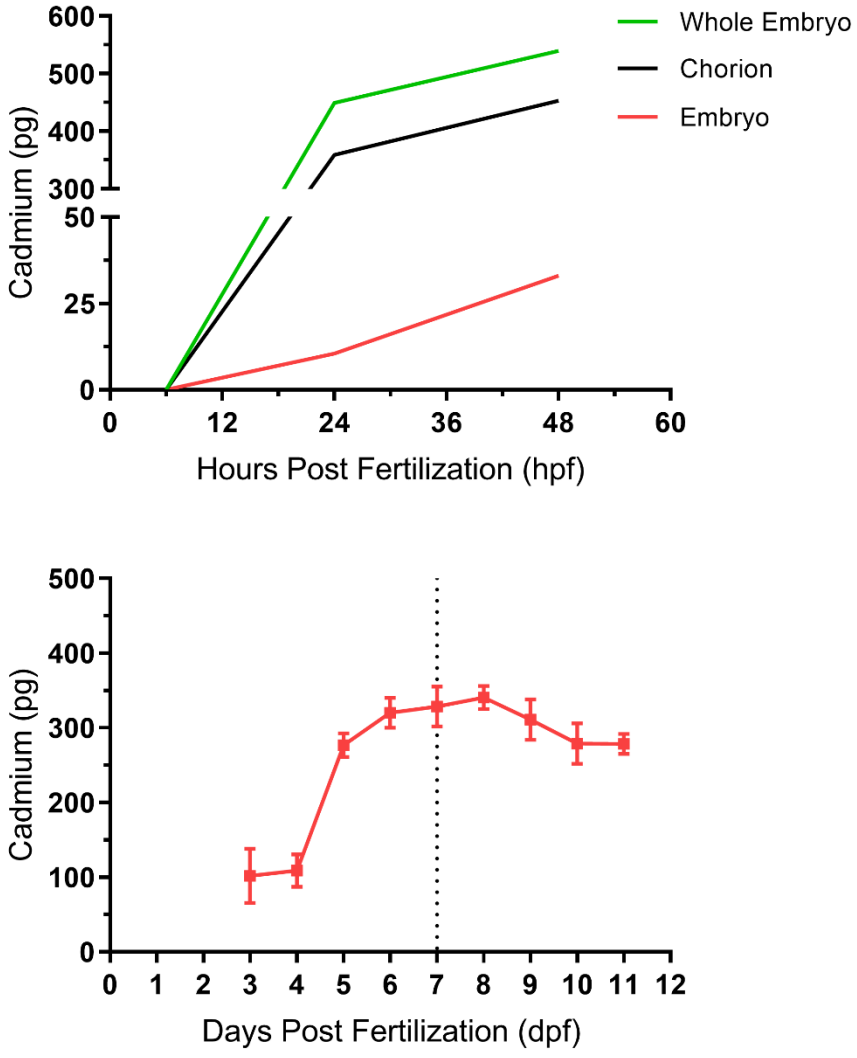

Figure 3: The chorion acts as a barrier against Cd uptake during the first 48 hpf.

Antioxidant treatment rescues Cd-induced hyperactivity

The Cd-induced increase in distance moved during dark periods is ameliorated with co-exposure to antioxidants. In this experiment zebrafish embryos were exposed to 40 ppb Cd alone, Cd + 1000 µg/L NAC, or Cd + 5 µg/mL AA form four hpf through five dpf. These antioxidant concentrations were chosen as they had no observed effect on larval development. Our results show that both NAC and AA are able to rescue the hyperactive phenotype induced by developmental Cd exposure (Fig 4). There is no significant difference in the distance moved in the dark for either NAC ($p = 0.46$) or AA ($p = 0.46$) compared to unexposed controls. These data show that the Cd-induced increase in distance moved in larval zebrafish can be rescued by antioxidant

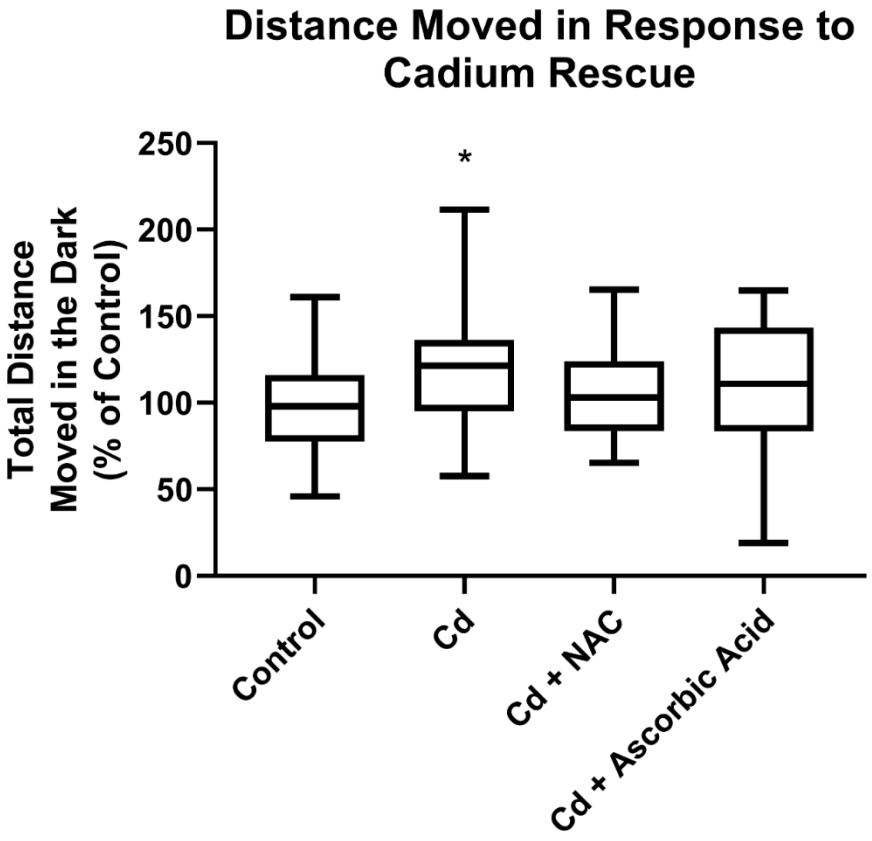

Figure 4: Total distance moved in the dark in Cd- and antioxidant-exposed zebrafish larvae at five dpf. NAC - n-Acetyl-Cysteine, * $p < 0.05$

Discussion

Our study indicates that developmental Cd exposure alters how larval zebrafish respond to visual stimuli by increasing their distance moved during dark periods (Fig 1). Our findings also show that this change in distance moved is threshold-dependent beginning at 30 ppb Cd (Fig 2). Co-exposure to 40 ppb Cd with either of the antioxidants NAC and AA were able to rescue the increase in distance moved observed in Cd exposure alone (Fig 3).

These data support the hypothesis that Cd-induced hyperactivity in larval zebrafish is ROS mediated and showed that this relationship is not concentration-dependent but instead threshold-dependent. Previous studies have shown a correlation between Cd exposure and behavioral changes along with increases in the expression of the antioxidant genes *sod*, *gpx*, and *cat* along with decreases in SOD and GPX protein activity. It is worth noting that Jin et al. showed that Cd exposure decreased distance moved in response to light-dark cycling even at their lowest concentration tested, 112 ppb Cd[10]. This is likely due to general Cd-induced toxicity as in our study, exposure to 60 ppb Cd results in increased edema and delays in swim bladder development. While other studies have shown that acute embryonic Cd exposure can decrease antioxidant protein levels and increase activity in adults, to our knowledge this is the first study to demonstrate that Cd-induced larval neurobehavioral changes are mitigated by antioxidant treatment indicating that ROS might be responsible for these observed behavioral changes.

As this neurobehavioral rescue was the result of both NAC and AA treatment the mechanism by which Cd induces ROS dependent behavioral changes remains unclear. AA is a very important aqueous phase antioxidant and at physiological pH, 99.9% of AA is present as $AscH^-$. $AscH^-$ is a donor antioxidant and reacts with radicals to produce the resonance stabilized tricarbonyl ascorbate free radical ($AscH^{\bullet}$, pK=−0.86), which is rapidly deprotonated to form of

$Asc^{\bullet-}$. Therefore the product of AA oxidation by many ROS is the semidehydroascorbate radical ($Asc^{\bullet-}$) considered to be a terminal, poorly reactive radical[26,27]. Additionally, AA cooperates with lipid soluble α-tocopherol, the most active form of Vitamin E, to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins[28]. NAC on the other hand provides cysteine, which is the rate limiting amino acid in GSH production[29]. GSH carries a free thiol group that acts as a reducing agent scavenging free radical or reducing superoxide radicals to hydrogen peroxide which can then be neutralized to water by catalase[30,31]. Considering the mechanism of action of NAC and AA our data suggest that the Cd-induced increase in distance moved occurs due to two potential mechanisms, but which of either is the major driver, is unclear. The first is Cd directly interacts with GSH resulting in GSH depletion and increased H_2O_2 and free radicals while the second is that Cd causes mitochondrial damage leading to greater H_2O_2 and free radical production. Additional pharmacological interventions and more direct measures could provide insight into which of these two mechanisms has the greatest impact on Cd-induced ROS-mediated hyperactivity. These might include the use of 4-hydroxy-2,3-trans-nonenal, a GPX inhibitor[32], or the catalase-specific inhibitor 3-amino-1,2,4-triazole[33]; gene expression to confirm that NAC and/or AA rescue ROS inducible genes; and direct assessment the zebrafish oxidative state using the respiratory burst assay[34].

Given the nature of Cd as a ubiquitous environmental pollutant and that the incidence of gestational exposure is common according to NHANES data from 2009 - 2016[35–38], together with the increase seen in neurodevelopmental disorders it is critical that research into the developmental effects of Cd continue. While Cd is not the only cause for this rise, the data we have presented here show that it is capable of causing neurobehavioral changes in larval zebrafish. In addition, further work to investigate the use of antioxidants through enriched diets

or supplementation to help reduce the negative behavioral effects caused by Cd exposure warrants further investigation.

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