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Gender Differences in Cardiac Remodeling Induced by a High-Fat Diet and Lifelong, Low-Dose Cadmium Exposure

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Abstract

Childhood obesity, which is prevalent in developed countries, is a metabolic risk factor for cardiovascular disease. Cadmium (Cd), a ubiquitous environmental toxic metal, also has deleterious effects on the cardiovascular system. However, the combined effects of a high-fat diet (HFD) and lifelong, low-dose Cd exposure on cardiac remodeling remain unclear. This study aims to determine the effects of combined HFD and Cd exposure on cardiac remodeling, as well as gender-specific differences in the response. C57BL/6J mice were exposed to Cd at a low dose (L-Cd, 0.5 ppm) or high dose (H-Cd, 5 ppm) via drinking water from conception to sacrifice. After being weaned, the offspring mice were fed with a HFD (42% kcal from fat) for an additional 10 weeks. H-Cd exposure significantly increased Cd accumulation in the hearts of both parents and their offspring; a HFD showed no added effects on cardiac Cd content. H-Cd exposure increased cardiac metallothionein protein levels only in female mice, regardless of dietary intake.

Histological analysis revealed that H-Cd exposure combined with a HFD induced cardiac hypertrophy and fibrosis only in female mice. This was further supported by elevated expression of ANP and COL1A1 protein levels along with COL1A1, COL1A2, and COL3A1 mRNA levels. Profibrotic markers PAI-1, CTGF, and FN were also elevated in HFD/H-Cd-exposed female mice. Levels of the oxidative stress marker 3-NT significantly increased in the hearts of HFD-fed female mice, whereas Cd exposure showed no additional effects. Of all the antioxidant markers examined, levels of CAT significantly increased in mice fed a HFD, regardless of gender and Cd exposure. In

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.8b00386. Body weight gain in male and female offspring mice; cardiac Cd content in the hearts of parental male and female mice (PDF)

The authors declare no competing financial interest.

summary, a HFD combined with lifelong, low-dose Cd exposure induces cardiac hypertrophy and fibrosis in female but not male mice, a response that is independent of oxidative stress.

Graphical Abstract

INTRODUCTION

Obesity is a growing health burden around the world, increasing nearly 3-fold since 1975. In 2016, more than 650 million adults, 340 million children or adolescents aged 5–19, and 41 million children younger than 5 were overweight or obese.¹ The marked rise in the prevalence of obesity, which is positively associated with a variety of metabolic and cardiovascular disorders,^{2,3} may be related to increased dietary fat consumption.

Previously, researchers mainly focused on the health effects of obesity in adults. In recent years, however, health issues related to childhood obesity have drawn increasing attention. Researchers created animal models that mimic childhood obesity to explore associations with cardiovascular disease in adulthood. We have recently demonstrated that feeding a high-fat diet (HFD, 60% kcal from fat) to 4 week old male C57BL/6J mice for 3 or 6 months induced overt cardiac hypertrophy.⁴ Xu et al.⁵ fed 6 week old male C57BL/6J mice with a similar HFD for 6 months, which resulted in cardiac dysfunction, hypertrophy, and fibrosis. However, the World Health Organization (WHO) recommends that saturated fats account for 10–30% of total energy intake.⁶ Therefore, a HFD with 60% kcal from fat far exceeds the WHO recommendation. It is known that a diet with 42% kcal from fat aligns with a typical Western diet and may be more representative of current lifestyles; thus, a similar Western diet has been widely used in research.^{7–9} Accordingly, we used a similar diet in the present study.

Cadmium (Cd) is a ubiquitous environmental toxicant metal, ranking seventh on the Agency for Toxic Substances and Disease Registry's Substance Priority List.¹⁰ Food is the major source of non-occupational Cd exposure in the general population, although cigarette smoking also plays a role and may contribute to Cd burden to a greater extent than food intake in heavy smokers. In 2010, the WHO established a provisional tolerable monthly intake of 25 $\mu\text{g}/\text{kg}$ for Cd.¹¹ Mounting human evidence suggests that Cd is a cardiovascular risk factor.^{12,13} Data from the National Health and Nutrition Examination Survey strongly suggested that Cd, even at low levels of exposure, remains an important determinant of cardiovascular disease mortality.¹³ The half-life of Cd in the human body ranges from 10 to 30 years. Reduction of the provisional tolerable weekly intake of Cd from 7 to 2.5 $\mu\text{g}/\text{kg}$ by the European Food Safety Authority in 2009 reflects evidence that low-dose Cd exposure

can also induce adverse health effects.¹⁴ After the discovery that the heart also accumulates Cd after low-dose exposure (5 ppm for 24 months) in rats,¹⁵ animal studies began to focus on low-dose and long-term Cd exposure and its related cardiac injury. Turdi et al.¹⁶ exposed adult male mice to Cd for 4 weeks (20 nmol/kg, i.p. every other day, about 28 μ g Cd/day), which induced overt cardiac interstitial fibrosis and impaired cardiac contractile function at 60 weeks. Mechanistically, the detrimental effects of Cd on the heart may be related to cardiac antioxidant system depression,¹⁷ impairment of mitochondrial respiration in cardiomyocytes,¹⁸ and reactive oxygen species (ROS) formation resulting from inhibition of the electron transfer chain.¹⁹

Due to the complexity of our environment, exposure to multiple health risks is common. A study in mice showed that Cd altered the serum lipid and cholesterol profile to a more atherogenic state.²⁰ Therefore, investigating the contribution of exposure to both a moderate HFD and low-dose Cd to obesity and associated metabolic cardiovascular complications may be more relevant to public health concerns. Turkcan et al.⁷ reported for the first time that the combination of high cholesterol and Cd levels (about 61 ppm of Cd via drinking water) for 12 weeks increased the risk of heart failure through cardiac fibrosis.

For the general population living in Western Europe, the United States, and Australia, the average daily oral intake of Cd by nonsmokers living in unpolluted areas is 10–25 μ g.²¹ Therefore, we utilize Cd doses (0.5 or 5 ppm, equivalent to about 3.5 or 35 μ g/day, respectively) which are closer to human environmental Cd exposure. Additionally, gender differences in this response have not been examined. Herein, we aim to investigate the effects of a moderate HFD (42% kcal from fat) combined with lifelong, low-dose Cd exposure on cardiac remodeling and to explore gender-specific differences in this response and their potential mechanisms.

MATERIALS AND METHODS

Animals and Procedures.

Ten week old parental male and female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). After 2 weeks, parental male and female mice were housed together to mate and were divided into three groups: a control (Ctl) group, a low-dose Cd (L-Cd, 0.5 ppm) group, and a high-dose Cd (H-Cd, 5 ppm) group; the Cd groups were exposed via drinking water containing CdCl₂ (0.815 mg/L for 0.5 ppm of Cd and 8.15 mg/L for 5 ppm of Cd). There were 10 parental female mice and 5 parental male mice for each group; all the parental mice were sacrificed after weaning (about 24 weeks old). After the offspring mice were born, they were exposed to the same dose of Cd as their parents. After being weaned, each group was subdivided into low-fat diet (LFD) (i.e., normal diet, 13% kcal from fat) and HFD (TD.09682, 42% kcal from fat, ENVIGO) groups, which means before 10 weeks of HFD exposure, mice were pre-exposed to Cd for an additional 6 weeks (3 weeks conception and 3 weeks lactation periods). A previous study chose 8 weeks as an observation time point for subacute Cd-induced cardiac injury.²² In our study, we chose a lower-fat HFD and lower-dose Cd; HFD exposure time remained at 10 weeks, but Cd exposure time was 16 weeks. Based on our previous data,⁹ feeding adult male mice a HFD (42% kcal from fat) for 12 weeks did not cause overt cardiac hypertrophy or

remodeling. However, whether Cd (0.5 or 5 ppm) exposure for 16 weeks alone or combined with exposure to 10 weeks of a HFD (42% kcal from fat) may show significant effects on cardiac remodeling remains to be elucidated. In summary, we had offspring male and female mice, and both genders of offspring mice were divided into six groups (n = 3–6 for each group): Ctl + LFD, Ctl + HFD, L-Cd + LFD, L-Cd + HFD, H-Cd + LFD, and H-Cd + HFD. After 10 weeks of HFD exposure, all of the mice were sacrificed, and their hearts were harvested for metal analysis, histopathological and protein analysis, and mRNA measurements. All of the mice were housed in shoebox cages with corn cob bedding at 22 °C with a 12 h light/dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care.

Materials.

CdCl₂ was purchased from Alfa Aesar (Tewksbury, MA, USA). The antibodies against atrial natriuretic peptide (ANP), collagen 1a1 (COL1A1), β -actin, catalase (CAT), superoxide dismutase 2 (SOD2), and NADPH dehydrogenase quinone 1 (NQO-1) were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). The antibodies for transforming growth factor β 1 (TGF- β 1) and fibronectin (FN) were from Abcam (Cambridge, MA, USA). Plasminogen activator inhibitor-1 (PAI-1) antibody was purchased from BD (Franklin Lakes, NJ, USA). The 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE) antibodies were from Millipore (Billerica, CA, USA) and Alpha Diagnostic International (San Antonio, TX, USA), respectively. Antibodies for metallothionein (MT) were from DakoCytomation (Carpinteria, CA, USA). All other chemicals were of the highest purity commercially available.

Cd Content in Cardiac Tissue.

Cd content in cardiac tissue was measured by ICP-MS (X series II, Thermo Fisher, Waltham, MA). All samples were digested by 1 mL of 70% nitric acid at 85 °C for 4 h. Samples were cooled at room temperature, centrifuged at 5000 rpm for 1 min, diluted into 34 mL doubly deionized water (2% nitric acid solution), vortexed, and assayed by ICP-MS.

Histological Analysis.

Excised heart tissue specimens were fixed in 10% formalin processed in graded alcohol and xylene and then embedded in paraffin. Paraffin blocks were sliced into sections of 5 μ m. To detect fibrosis or collagen accumulation in tissues, sections underwent Sirius Red staining. Sections were also stained with fluorescein-conjugated wheat germ agglutinin (WGA) (Alexa Fluor-488, Invitrogen) to evaluate the cross-sectional myocyte areas. The stained sections were then viewed using the Nikon Eclipse E600 microscope (Tokyo, Japan) and assessed in 5–10 fields of view for each heart using ImageJ 1.44 software (Media Cybernetics, Bethesda, MD).

Western Blot (WB) Analysis.

WB was performed according to protocols described in detail in our previous studies.⁹ The first antibodies were diluted in different concentrations: from 1:500 to 1:3000. MT

expression was detected with a modified WB protocol based on the previous protocol:²³ prepare a 20 μ L sample (protein loading 30 μ g), add dithiothreitol from a 0.5 M stock solution to a final concentration of 5 mM, and incubate for 30 min at room temperature to reduce disulfide bonds. Then add iodoacetamide to a final concentration of 14 mM and incubate for 30 min at room temperature in the dark to alkylate cysteines. Next, quench unreacted iodoacetamide by adding 0.5 M dithiothreitol to an additional 5 mM and incubate for 30 min at room temperature in the dark. After adding a loading buffer, heat the sample at 95 °C for 5 min. Run 16% SDS-PAGE gel at 100 V until the tracking dye reaches the bottom. Then incubate the gel in the transfer buffer (15% methanol with 2 mM CaCl_2) for 10 min. Transfer the proteins to a nitrocellulose membrane (0.2 μ m) at 4 °C using the same transfer buffer. Cut the membrane into two pieces across the markers at 25–30 kDa; the upper one is for β -actin, and the lower one is for MT. The upper membrane is following the regular WB protocol; however, the lower membrane should be incubated in 2.5% glutaraldehyde for 60 min in the dark. Wash the lower membrane for 5 min with 1 $\times$ phosphate-buffered saline (PBS) two times and 5 min with 1 $\times$ PBS containing 50 mM ethanolamine one time, which will quench the residual glutaraldehyde. Block the membrane in 1 $\times$ PBS with 3% bovine serum albumin at room temperature for 60 min. Incubate the membrane in 1:1000 Dako anti-MT monoclonal antibody in 1 $\times$ PBS with 3% bovine serum albumin at 4 °C overnight and then follow the regular WB procedure.

Reverse Transcription-Polymerase Chain Reaction (RT-qPCR).

The RT-qPCR procedure has been described before.⁹ The following primer sets were used to perform PCR: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mm99999915_g1; ANP, Mm01255747_g1; myosin heavy chain β (β -MHC), Mm00600555_m1; COL1A1, Mm00801666_g1; collagen 1a2 (COL1A2), Mm0048388_m1; collagen 3a1 (COL3A1), Mm00802300_m1; connective tissue growth factor (CTGF), Mm01192933_g1; and TGF- β 1 Mm00446190_m1. The primer sets for PCR were obtained from Thermo Fisher (Grand Island, NY, USA).

Statistical Analysis.

Experimental data were presented as mean $\pm$ standard deviation (SD) ($n = 3$ –6 per group). Statistical analyses were carried out using a one-way ANOVA with Tukey post-hoc test using Prism 7.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

HFD Feeding Induced Obesity, but Cd Exposure Did Not Significantly Affect Body Weight: H-Cd Exposure Significantly Increased Parental Cd Content in the Heart.

Lifelong, low-dose exposure to Cd did not affect body weight in either male or female mice (Figure S1A–D). HFD feeding for 4 weeks in male mice and 2 weeks in female mice significantly increased body weight. However, neither L-Cd nor H-Cd exposure significantly affects HFD-induced body weight gain. H-Cd, but not L-Cd, significantly increased Cd accumulation in the hearts of parental mice; additionally, parental females accumulated significantly more (nearly 5-fold) cardiac Cd than parental males (Figure S2A,B).

H-Cd Exposure Significantly Increased Offspring Mice's Cardiac Cd Accumulation but Only Increased MT Protein Expression in Female Mice, Regardless of Dietary Conditions.

In the offspring, H-Cd exposure significantly increased the Cd content in the heart, without a difference between male and female mice. HFD feeding had no effect on cardiac Cd content, regardless of the dose of Cd exposure (Figure 1A,B).

Prior research showed that MT plays an important role in Cd disposition and detoxification. Therefore, in the present study, we also detected MT protein level using a WB assay. No change in cardiac MT protein expression was seen in male mice exposed to either L-Cd (Figure 1C) or H-Cd (Figure 1D). In contrast, MT protein level was significantly upregulated in female mice exposed to H-Cd instead of L-Cd (Figure 1E,F). HFD feeding had no significant effect on cardiac MT expression (Figure 1C–F).

HFD Feeding Combined with H-Cd Exposure Induced Cardiac Hypertrophy and Fibrosis in Female Mice but Not in Male Mice.

WGA staining (Figure 2A,C) and its quantification analysis (Figure 2B,D) revealed that neither HFD feeding nor Cd exposure alone affected myocyte size. However, HFD feeding combined with H-Cd exposure induced overt cardiac hypertrophy in female mice but not in male mice. ANP protein (Figure 2E–H) and β -MHC mRNA (Figure 2I,J) levels, which are molecular hypertrophic markers, were also upregulated in HFD/H-Cd-exposed female mice, confirming the WGA staining observations.

Sirius Red staining was used to measure collagen accumulation in the heart, and semiquantitative analysis revealed that HFD feeding combined with H-Cd exposure significantly increased collagen accumulation in female mice (Figure 3A–D). It is known that the cardiac extracellular matrix is mainly composed of collagen 1 (80%) and collagen 3 (20%);²⁴ collagen 1 consists of two isoforms, COL1A1 and COL1A2. We next performed WB for COL1A1 with the only available antibody (Figure 3E–H). It showed that HFD feeding marginally increased COL1A1 protein expression in female mice ($P = 0.058$, Figure 3H), and the exposure to H-Cd increased HFD-induced COL1A1 protein expression in female mice (Figure 3H). There was no significant effect of either exposure to Cd or HFD on cardiac COL1A1 protein expression in male mice (Figure 3E,F). To further confirm the WB results for COL1A1 and also to explore other isoforms of collagen 1 and collagen 3, RT-qPCR was applied to determine mRNA levels of COL1A1, COL1A2, and COL3A1. Results showed that HFD/H-Cd-exposed female mice exhibited significantly increased COL1A1, COL1A2, and COL3A1 mRNA levels (Figure 4B,D,F); such responses were not observed in male mice (Figure 4A,C,E).

Effects of HFD Feeding and Cd Exposure on Cardiac Profibrotic Cytokines PAI-1, TGF- β 1, FN, and CTGF.

PAI-1, TGF- β 1, FN, and CTGF are prominent cytokines known to be implicated in the development of cardiac fibrosis. To elucidate the roles of these cytokines in cardiac changes induced by HFD feeding and H-Cd exposure, PAI-1, TGF- β 1, and FN protein or mRNA levels were measured through WB assay or RT-qPCR (Figure 5). Consistent with the phenotype of cardiac fibrosis, PAI-1 and FN protein levels (Figure 5F,H) and CTGF mRNA

levels (Figure 5J) were all significantly elevated in female mice in the HFD/H-Cd group. Interestingly, FN levels were also significantly elevated in female mice in the HFD/L-Cd group (Figure 5E,G). and TGF- β 1 protein and mRNA levels remained comparable across groups of female mice (Figure 5E–J).

HFD Feeding, Not Cd Exposure, Induced Elevated Expression of 3-NT in Female Mice but Had No Effect on 4-HNE Expression.

As numerous previous animal experiments have demonstrated both HFD feeding and Cd exposure can induce cardiac oxidative stress,^{25–28} the present study tested ROS/reactive nitrogen species (RNS) production and some well-known antioxidant markers. Accumulation of 3-NT has been used as a biomarker of RNS formation and 4-HNE as a marker of lipid peroxidation, so we investigated the mechanism of this effect by using 3-NT and 4-HNE to determine the oxidative stress status in the hearts of these mice.

Results suggested that only HFD feeding upregulated 3-NT protein expression in female mice, regardless of Cd exposure status (Figure 6C,D). Consistent with other markers, the 3-NT level showed no response to HFD feeding and Cd exposure in male mice (Figure 6A,B). In contrast to 3-NT, 4-HNE protein levels were unaffected by HFD feeding and Cd exposure in both male and female mice (Figure 7A–D).

To further explore antioxidant status, we measured the protein expression of CAT, SOD2, and NQO-1 by WB assay (Figure 8A–H). Only CAT protein levels were significantly increased in HFD-fed male and female mice, independent of Cd exposure (Figure 8A–D).

DISCUSSION

In the present study, we observed that HFD feeding in young mice induced obesity, whereas both L-Cd and H-Cd exposure showed no added effects on body weight (Figure S1A–D). Moreover, only H-Cd exposure increased offspring's Cd burden in the heart, which was independent of gender. Interestingly, MT protein level was only upregulated in H-Cd-exposed female mice but not in male mice. Furthermore, HFD feeding for 10 weeks did not induce any overt cardiac remodeling, which was consistent with our previous study.⁹ However, after coexposure of HFD and H-Cd, female mice exhibited cardiac hypertrophy and fibrosis, an effect not seen in male mice. In addition, HFD feeding elicited a protein nitration increase in female mice but not males, with no effect on lipid peroxidation level. Lifelong exposure to low-dose Cd did not increase either protein nitration or lipid peroxidation.

In recent decades, exposure to environmental toxic metals, such as arsenic, lead, Cd, and mercury, has become a global public health issue. Exposure to Cd includes occupational and non-occupational exposure. Occupational exposure mainly consists of respiratory inhalation and skin exposure. Whereas non-occupational exposure is mainly through air, water, and food, dietary intake is the main source of exposure. Smoking further increases Cd exposure, as every cigarette contains 0.5–2 μ g of Cd.²⁹ Inhalation may result in substantially higher Cd absorption rates than ingestion (5–50% vs 1–10%, respectively).⁷ According to the International Cd Association, the expected amount of Cd in suspended particles varies from

0.1 to 5 ng/m³ in rural areas and from 15 to 150 ng/m³ in industrial areas (1 ng/m³ = 1 × 10⁻⁹ ppm).²⁹ The daily intake of Cd in the most heavily contaminated areas reaches 600–2000 µg/day.²¹ Generally, the acceptable level of Cd intake from food and other agricultural products per month is 25 µg/kg.¹¹

In the present study, we used an animal model more closely approximating humans' dose of natural exposure to Cd (10–25 µg/day), as well as exposure route (drinking water). Turkcan et al.⁷ have reported that combined exposure to high cholesterol (45% kcal from fat) and Cd for 12 weeks increases the risk of heart failure through cardiac fibrosis in ApoE^{-/-} mice. However, our recent study found that a HFD (42% kcal from fat) fed to adult male C57BL/6J mice for 12 weeks did not cause overt cardiac hypertrophy or fibrosis.⁹ Additionally, Ceylan et al.⁸ found that 4 week old male mice consuming a HFD (42% kcal from fat) for 6 months had impairments in myocardial geometry, morphology (cardiac hypertrophy and interstitial fibrosis), contractile function, and intracellular Ca²⁺ handling. These studies indicate that 10 weeks of exposure to a HFD (42% kcal from fat) is inadequate to induce cardiac remodeling; however, we hypothesized that coexposure to Cd may show some synergistic effects. Consistent with our hypothesis, our results confirmed that the 6 week pre-exposure and 10 week coexposure to low-dose Cd increased susceptibility of mice to cardiac injury induced by 10 week exposure to a HFD (42% kcal from fat).

MT, as a ubiquitous, cysteine-rich (30%), metal-binding protein, can bind 7 g atoms of divalent metals like zinc and Cd.³⁰ Many studies have shown the important role of the Cd–MT complex in disposition and elimination of Cd in the body. For instance, transgenic mice with overexpressed MT showed that MT did not inhibit intestinal Cd absorption or affect initial Cd distribution to various tissues.^{31,32} Compared with nontransgenic mice, however, MT-transgenic mice showed reduced elimination of Cd from the liver; a Cd–MT complex was formed in the liver and then slowly released into the circulation and later delivered to the kidneys.^{31,32} The formation of a Cd–MT complex resulted in detoxifying Cd by preventing free Cd reactions with cytosol-critical organelles.³³

The formation of a Cd–MT complex is a good way to reduce the toxicity of acute Cd at relatively high doses; however, whether the formation of a Cd–MT complex in the organ also increases a mild but chronic inflammatory response which would induce injury in the organ remains an interesting question. To the best of our knowledge, this is the first report of a gender difference in cardiac remodeling after combined exposure to a HFD and Cd in mice. Adding new evidence to the literature, we found here that both male and female mice showed similar degrees of cardiac Cd accumulation in H-Cd exposure groups; however, increased cardiac MT expression was only found in H-Cd-exposed female mice, regardless of dietary conditions (Figure 1); interestingly, combined exposure to a HFD and H-Cd caused mild cardiac remodeling, such as hypertrophy and fibrosis, only in female mice (Figures 2–4). Progress in understanding sex-specific physiopathology is still scarce. Most preclinical and clinical studies were carried out in one sex (mainly males), with results extrapolated to the other. Studies predominantly used males due to a belief that studies with females had higher variability due to the changes in sex hormones during the reproductive cycle.³⁴ So far, the exact reason why MT only increased in H-Cd-exposed female mice remains elusive; likewise, it remains unclear why a HFD combined with H-Cd induced

cardiac remodeling only in female mice. Further studies are needed to reveal the underlying mechanisms of sex differences observed in the response to combined exposure to a HFD and H-Cd.

Ample evidence suggests that cardiac hypertrophy is an adaptive response initially capable of compensating for the increased myocardial workload in an effort to maintain normal cardiac function; however, with persistent fat diet intake and adiposity, physiological cardiac remodeling often develops into maladaptive pathological hypertrophy, resulting in progression of cardiac contractile dysfunction and, ultimately, heart failure.³⁵⁻³⁷ In the present study, we found that HFD/H-Cd coexposure induced significant cardiac hypertrophy and fibrosis in female mice, suggesting that pathological hypertrophy and remodeling was induced. It is known that synthesis of both type 1 and type 3 collagen is markedly increased in the remodeling fibrotic heart regardless of the etiology of fibrosis.²⁴ Collagen 1 exhibits more intense and prolonged upregulation than collagen 3.³⁸ Consistent with these findings, we confirmed the presence of cardiac fibrosis shown by high expression of COL1A1 protein levels, as well as COL1A1, COL1A2, and COL3A1 mRNA levels. Unlike other organs, the heart has very limited regenerative capacity following injury; instead, repair processes involve the removal of necrotic cardiomyocytes followed by fibrotic scar tissue replacement that acts to preserve myocardial structural and functional integrity.³⁹ In general, the differentiation of cardiac fibroblasts to more active myofibroblasts is the hallmark of cardiac fibrosis. In the process of myofibroblast activation, high levels of cytokines (e.g., interleukin-6), TGF- β 1, FN extra-domain A, and mechanical stress that have accumulated within the damaged area promote differentiation of active myofibroblasts.⁴⁰

In the present study, PAI-1 and FN protein levels, along with CTGF mRNA levels, were significantly increased in HFD/HCd-exposed female mice, whereas both TGF- β 1 protein and mRNA levels were unchanged independent of gender, diet, or metal status. This finding is consistent with those of Turkcan et al.'s study,⁷ who showed that although the combination of Cd and a high cholesterol level increased the risk for heart failure through cardiac fibrosis, the TGF- β 1 levels in the heart remained unchanged. In mammals, among the three isoforms of TGF- β (TGF- β 1, 2, and 3), TGF- β 1 is the predominant isoform in the cardiovascular system. TGF- β 1 is present in the normal heart as a latent complex, unable to interact with its receptors. Following cardiac injury, latent TGF- β 1 is converted to the active form, and activation of a relatively small amount of latent TGF- β 1 is sufficient to induce a maximal cellular response.⁴¹ TGF- β 1 activation induces myofibroblast transdifferentiation.⁴² However, in both Turkcan et al.'s⁷ and our own studies here, TGF- β 1 levels remained unchanged even though the CdCl₂ exposure dose and time varied widely from 8.15 mg/L for 16 weeks in the present study to 100 mg/L for 12 weeks in Turkcan et al.'s study; however, cardiac fibrosis was seen, suggesting the existence of other responsible mechanisms. It is known that the presence of fibrosis is also influenced by the balance of matrix metalloproteinases and their inhibitors.⁴³ In addition, macrophages and other cell types can also cause fibrosis independent of TGF- β 1 action;⁴⁴ therefore, chronic Cd exposure at very low doses may induce cardiac fibrosis independent of TGF- β 1 stimulation and dependent on other profibrotic mechanisms.

Although Cd is a nonredox metal, one of the important mechanisms underlying its toxicity is the induction of oxidative stress stemming from increased generation of ROS and RNS and/or depletion of the antioxidant defense system. Reportedly, Cd can replace Fe in various cytoplasmic and membrane proteins, such as ferritin and apoferritin, and thus increase the amount of freely available Fe ions that participate in Fenton reactions and generate ROS.⁴⁵ Cd also generates free radical formation by increasing the intracellular calcium level.⁴⁶ Cd-induced oxidative stress may also contribute to its impairment of the antioxidative defense system in cells or tissues because Cd has a high affinity for –SH groups in enzymes of the antioxidative defense system, such as SOD, CAT, glutathione peroxidase, and glucose-6-phosphate dehydrogenase, and subsequently inhibits their activities.⁴⁷ Animal studies have shown that Cd can induce cardiac oxidative damage and that antioxidant treatment can ameliorate these detrimental effects.^{27,28} In the present study, L-Cd and H-Cd exposure neither increased nitrosative or oxidative damage, as determined by protein nitrosative marker 3-NT and lipid peroxidation marker 4-HNE, nor suppressed the antioxidative system, reflected by multiple antioxidant markers (Figure 8). This may be due to the levels of Cd exposure, which were lower than those used in prior studies.^{27,28} It is worth mentioning that Cd exposure even had no effect on the HFD-increased RNS level in female mice (Figure 6C,D). Of all the antioxidant markers measured here, CAT protein levels were significantly elevated in HFD-fed male and female mice. CAT, a common enzyme found in nearly all living organisms exposed to oxygen, catalyzes hydrogen peroxide to water and oxygen; therefore, it is a very important enzyme in protecting the cell from oxidative damage caused by ROS. Likewise, CAT has very high catalytic efficiency; one CAT molecule can convert millions of hydrogen peroxide molecules to water and oxygen.⁴⁸ We assume that the increased CAT levels may explain why there was no peroxidation damage, such as 4-HNE accumulation, whereas HFD-induced 3-NT accumulation was not attenuated by increased CAT, as illustrated in Figure 9.

In summary, the combination of a moderate HFD and lifelong, low-dose Cd exposure induces cardiac hypertrophy and fibrosis in female mice, even though either exposure alone does not cause significant pathological changes in the heart. This suggests that we should avoid exposure to multiple health risk factors even at relatively low, nontoxic levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HFD	high-fat diet
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LFD	low-fat diet
Cd	Cadmium
WHO	World Health Organization
L-Cd	low-dose Cd
H-Cd	high-dose Cd
ANP	atrial natriuretic peptide
COL1A1	collagen 1a1
CAT	Catalase
SOD2	superoxide dismutase 2
NQO-1	NADPH dehydrogenase quinone 1
TGF-β1	transforming growth factor β 1
FN	fibronectin
PAI-1	Plasminogen activator inhibitor-1
3-NT	3-nitrotyrosine
4-HNE	4-hydroxynonenal
MT	metallothionein
WGA	wheat germ agglutinin
WB	Western blot
RT-qPCR	reverse transcription-polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
β-MHC	myosin heavy chain β
COL1A2	collagen 1a2
COL3A1	collagen 3a1
CTGF	connective tissue growth factor
SD	standard deviation
ROS	reactive oxygen species
RNS	reactive nitrogen species

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Figure 1.

Cd content in the hearts of offspring mice. H-Cd induced significantly increased MT expression in female but not male mice, which was not affected by HFD. Cd content in the hearts of male (A) and female (B) mice. MT protein expression in the hearts of male (C,D) and female (E,F) mice determined by Western blot assay. Data shown in graphs represent mean $\pm$ SD, $n = 3-6$ for each group. * $P < 0.05$ vs Ctrl + LFD group; \$ $P < 0.05$ vs Ctrl + HFD group; # $P < 0.05$ vs L-Cd + LFD; & $P < 0.05$ vs L-Cd + HFD; + $P < 0.05$ vs H-Cd + LFD. ♂ means male; ♀ means female.

Figure 2.

HFD combined with H-Cd elicited overt cardiac hypertrophy in female mice. (A,C) Cardiac tissue WGA staining and (B,D) quantification of myocyte cross-sectional area (40×, and scale bar = 50 μm). (E-H) ANP protein levels after exposure to HFD and different doses of Cd in males and females were determined by Western blot assay. β-MHC mRNA levels were measured via RT-qPCR in male (I) and female (J) mice. Data shown in graphs represent mean ± SD, *n* = 3–6 for each group. **P* < 0.05 vs Ctl + LFD group; [§]*P* < 0.05 vs Ctl + HFD

group; [#] $P < 0.05$ vs L-Cd + LFD; [&] $P < 0.05$ vs L-Cd + HFD; ⁺ $P < 0.05$ vs H-Cd + LFD. ♂ means male; ♀ means female.

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Figure 3.

H-Cd combined with HFD induced cardiac fibrosis in female mice. Collagen accumulation in the heart was measured by Sirius Red staining in male (A) and female (C) mice (collagen is red; 20 $\times$, scale bar = 100 μ m) and its quantitative analysis results in male (B) and female mice (D). (E-H) Collagen 1A1 protein levels after exposure to HFD and different doses of Cd in males and females were determined by Western blot assay. Data shown in graphs represent mean $\pm$ SD, $n = 3-6$ for each group. * $P < 0.05$ vs Ctl + LFD group; $^{\$}P < 0.05$ vs

Ctl + HFD group; [#] $P < 0.05$ vs L-Cd + LFD; [&] $P < 0.05$ vs L-Cd + HFD; ⁺ $P < 0.05$ vs H-Cd + LFD. ♂ means male; ♀ means female.

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Figure 4.

Collagen 1 and collagen 3 mRNA expression increased significantly in HFD/H-Cd-exposed female mice. COL1A1 (A,B), COL1A2 (C,D), and COL3A1 (E,F) mRNA levels after exposure to HFD and different doses of Cd in males and females were measured by RT-qPCR. Data shown in graphs represent mean $\pm$ SD, $n = 3-6$ for each group. * $P < 0.05$ vs Ctl + LFD group; \$ $P < 0.05$ vs Ctl + HFD group; # $P < 0.05$ vs L-Cd + LFD; & $P < 0.05$ vs L-Cd + HFD; + $P < 0.05$ vs H-Cd + LFD. ♂ means male; ♀ means female.

Figure 5.

Effect of Cd and HFD coexposure on some well-known profibrotic markers. PAI-1, TGF- β 1, and FN protein expression was detected via Western blot assay (A–H). β -Actin was used for loading control. RT-qPCR was performed to measure CTGF and TGF- β 1 mRNA levels (I, J). Data shown in graphs represent mean $\pm$ SD, $n = 3$ –6 for each group. * $P < 0.05$ vs Ctl + LFD group; $^{\$}P < 0.05$ vs Ctl + HFD group; $^{\#}P < 0.05$ vs L-Cd + LFD; $^{\&}P < 0.05$ vs L-Cd + HFD; $^{+}P < 0.05$ vs H-Cd + LFD. σ means male; ρ means female.

Figure 6.

HFD significantly increased 3-NT expression in female mice, regardless of Cd exposure status. 3-Nitrotyrosine (3-NT) was used as a biomarker of RNS formation. Western blot was performed to detect 3-NT protein levels in the present study. (A–D) Representative Western blot bands and their quantitative results for 3-NT. Data shown in graphs represent mean $\pm$ SD, $n = 3$ –6 for each group. * $P < 0.05$ vs Ctl + LFD group; $^{\$}P < 0.05$ vs Ctl + HFD group; $^{\#}P < 0.05$ vs L-Cd + LFD; $^{\&}P < 0.05$ vs L-Cd + HFD; $^{+}P < 0.05$ vs H-Cd + LFD. σ means male; ϕ means female.

Figure 7.

Neither HFD nor H-Cd showed any effects on 4-hydroxynonenal (4-HNE) protein levels. 4-HNE protein adducts were used as a marker of oxidant stress. Western blot assay was performed to detect 4-HNE protein levels in the present study. (A–D) Representative Western blot bands and their quantitative results for 4-HNE. Data shown in graphs represent mean $\pm$ SD, $n = 3-6$ for each group. * $P < 0.05$ vs Ctl + LFD group; $^{\$}P < 0.05$ vs Ctl + HFD group; $^{\#}P < 0.05$ vs L-Cd + LFD; $^{\&}P < 0.05$ vs L-Cd + HFD; $^{+}P < 0.05$ vs H-Cd + LFD. ♂ means male; ♀ means female.

Figure 8.

HFD significantly increased CAT protein expression in both male and female mice, with no added effects from L-Cd and H-Cd. To further confirm the role of oxidative stress in HFD/H-Cd-induced cardiac injury, several antioxidant markers were measured by Western blot assay, including CAT and SOD2 (A–D) and NQO-1 (E–H). (A–H) Representative Western blot bands and their quantitative results for the above markers. Data shown in graphs represent mean $\pm$ SD, $n = 3$ –6 for each group. * $P < 0.05$ vs Ctl + LFD group; $^{\$}P <$

0.05 vs Ctl + HFD group; [#] $P < 0.05$ vs L-Cd + LFD; [&] $P < 0.05$ vs L-Cd + HFD; ⁺ $P < 0.05$ vs H-Cd + LFD. ♂ means male; ♀ means female.

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Figure 9.

Working hypothesis. Catalase (CAT), a common enzyme found in nearly all living organisms exposed to oxygen, catalyzes hydrogen peroxide to water and oxygen; therefore, it is a very important enzyme in protecting cells from oxidative damage by hydrogen peroxide. CAT has very high catalytic efficiency; one CAT molecule can convert millions of hydrogen peroxide molecules to water and oxygen. Therefore, we assume that the increased CAT may explain why there was no peroxidation damage, such as 4-HNE accumulation, whereas HFD-induced 3-NT accumulation in females was not attenuated by increased CAT.