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Chronic Exposure to Arsenic and High Fat Diet Induces Sex-Dependent Pathogenic Effects on the Kidney

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Abstract

Both obesity and arsenic exposure are global public health problems that are associated with increased risk of renal disease. The effects of whole-life exposure to environmentally relevant levels of arsenic within dietary high fat diet on renal pathogenesis were examined. In this study, C57BL/6J mice were parentally exposed to 100ppb arsenic before conception. After weaning, both male and female offspring were maintained on 100ppb arsenic and fed either a normal (LFD) or high fat diet (HFD). At 10 and 24 weeks of age, the offspring were sacrificed and kidneys collected. Exposure to arsenic led to an increase body-weight in HFD diet-fed female but not male mice. Histological analysis shows that arsenic exposure significantly increases HFD-induced glomerular area expansion, mesangial matrix accumulation and fibrosis compared to LFD control animals. HFD alone increases renal inflammation and fibrosis; reflected by increases in IL-1 β , ICAM-1 and fibronectin levels. Arsenic exposure significantly increases HFD-induced inflammatory and oxidative stress responses. In general, male mice have more severe responses than female mice to HFD or arsenic treatment. These results demonstrate that arsenic exposure causes sex-dependent alterations in HFD-induced kidney damage.

Keywords

Arsenic; kidney injury; environmental contamination; obesity; inflammation

Introduction

Obesity is a growing problem in both developed and developing countries [1]. In the United States, more than 33% of adults and ~ 17% of adolescents are obese [2]. Obesity is associated with glucose intolerance, insulin resistance and dyslipidemia, as well as an

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increased risk for the development of chronic kidney disease [2]. The development of chronic kidney disease in the obese is also influenced by other factors including environmental toxicants, such as bisphenol, cadmium and arsenic [3].

The metalloid arsenic (As) is a common environmental toxicant, found in drinking water throughout the world [4, 5]. It is ranked number one on the Agency for Toxic Substances and Disease Registry's list of environmental chemical hazards and is classified as a group 1 carcinogen by the International Agency for Research on Cancer [6, 7]. Long-term exposure to arsenic-contaminated water and food can lead to metabolic dysfunction in organs that accumulate this metalloid, including the kidney. There are strong associations between markers of renal injury and chronic kidney disease progression with arsenic exposure [8–10].

Concomitant exposure of arsenic and high fat diet (HFD) in animal models is associated with the development of several diseases [11–13]. High fat diet enhances arsenic accumulation in liver and increases liver fibrosis [14, 15]. Co-exposure of HFD and arsenic synergistically increases oxidative stress in rat heart [14]. There is a scarcity of studies that consider the toxicological consequences of life-long or multigenerational arsenic exposure. Epidemiological and animal model studies indicate that *in utero* arsenic exposure is associated with an increased risk of developing adult diseases. The underlying mechanisms however, are still not well understood. Additionally, renal pathologies associated with whole life arsenic exposure in combination with HFD have not been investigated.

In the current study, the effects of whole-life exposure to an environmentally relevant level of inorganic arsenic on mouse kidney were examined. Additionally, the influences of HFD on arsenic-induced renal pathologies were determined. These studies addressed the following questions: (1) Does whole-life exposure to a low dose arsenic lead to renal pathologies? (2) Does whole-life exposure to arsenic alter the pathologies associated with HFD-induced renal damage? and (3) Are there gender specific differences in the responses to arsenic or HFD?

Materials and Methods

Animals and Exposures

All animal procedures followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee. Mice were maintained on a 12 h light/dark cycle at 25°C. Food and deionized water were provided ad libitum. After one week of acclimation, the diet of six-week-old male and female C57BL/6J mice was changed from standard laboratory chow to AIN-76A purified diet (Envigo TD 160377) to limit the confounding effects of metal contamination found in standard chow [16]. Body weight and water consumption were measured weekly throughout the study.

Arsenic exposure for male and female parental mice (F₀) began at 10 weeks of age. Arsenic containing drinking water was prepared from stock solutions of sodium meta(arsenite) in deionized water to a final concentration of 100 ppb. The concentration of arsenic used in this

study is within the ranges reported in drinking water supplies throughout the world [17, 18]. Arsenic-containing water was prepared twice weekly to minimize metalloid oxidation.

At 12 weeks, mice were placed into breeding triplets (1 male to 2 females) within each exposure group. During weaning dams were continuously exposed to arsenic. After weaning, the offspring (F₁) were exposed to the same concentration of arsenic as their parents until sacrifice. Additionally, offspring were fed either a low-fat (Envigo TD 160377 – 13% fat, Madison, WI) or high-fat (Envigo TD 09682 – 42% fat, Madison, MI) diet. Offspring were sacrificed 10 (N=8) or 24 (N= 3–8) weeks after weaning (Suppl. Fig. 1).

Mice were anesthetized with ketamine/xylazine and blood was collected from the vena cava prior to sacrifice via exsanguination, centrifuged (10,000 x g for 15 min at 4°C) and citrated plasma stored at –80°C until analyzed. Whole kidneys were collected, then snap-frozen in liquid nitrogen, processed for RNA isolation or fixed in 10% formalin for histology and immunohistochemistry.

Histopathological Analysis

Fixed renal samples (whole kidney) were ethanol dehydrated, embedded in paraffin and sliced into 5 µm sections for periodic acid Schiff (PAS) staining using standard protocols [19]. Glomerular volume and mesangial expansion degree were determined from PAS-stained sections. Digital images of random glomeruli were obtained and analyzed by two independent observers. Mesangial matrix expansion was defined by increased amounts of PAS positive material in the mesangial region. The mesangial matrix expansion area was determined from the mean area of each glomerulus.

Paraffin sections were also stained with Picro Sirius Red for quantitative analysis of fibrosis [19]. Tissue containing collagen stains red while normal tissue stains green.

For immunohistochemical staining, kidney sections were incubated with primary antibodies to 4-hydroxynonenal (4-HNE, Alpha Diagnostic International) or fibronectin (Abcam) overnight at 4 °C. Sections were then washed with PBS containing 0.1% Triton and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After the final wash with PBS/triton, sections were incubated with diaminobenzidine to visualize antigen-antibody complexes. ImageJ software was used for all quantitative measurements and subsequent calculations [20]

Western Blotting

Western blotting was performed as previously described [21]. Briefly, whole kidney tissue was homogenized in lysis buffer and then proteins collected following centrifugation. Protein concentrations were determined by Bradford assay [22]. Proteins were diluted in loading buffer, heated at 98°C for 5 min and then resolved following electrophoresis using 8% or 10% SDS-PAGE gels. After electrophoresis, proteins were transferred at 4°C to nitrocellulose membranes, then rinsed in Tris-buffered saline (TBS), incubated in blocking buffer (TBS, 5% milk and 0.5% bovine serum albumin) for 1 h and then washed three times with TBS containing 0.1% Tween-20. Membranes were incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with appropriate secondary

peroxidase-conjugated antibody for 1h at room temperature. Antigen-antibody complexes were visualized using an ECL kit (Bio-Rad, Hercules, CA). The protein content was measured using Image Lab software (Bio-Rad).

The primary antibodies used in the study were: Nrf2, fibronectin, collagen-1 and tumor necrosis factor α (TNF- α) from Abcam, Cambridge, MA (1:1,000 dilution); intercellular adhesion molecule-1 (ICAM-1), superoxide dismutase-2 (SOD-2), catalase (CAT), β -actin and IL-1 β from Santa Cruz Biotechnology, Santa, CA (1:1,000–5,000 dilution) and p38 MAPK and phospho-p38 MAPK (P-p38 MAPK) from Cell Signaling Technology, Danvers, MA (1:1,000 dilution).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA concentration and purity were assessed using a NanoDrop ND-1000 spectrophotometer. Quantitative Real-Time PCR was performed as previously described using an ABI 7300 Real-Time PCR system [23]. Primers targeting HO1, SOD2, NQO1, FN1 GAPDH were purchased from Thermo Fisher (Grand Island, NY). Data are expressed as fold differences compared to LFD controls, using the $\Delta\Delta$ Ct method and GAPDH as a reference gene [24].

Statistical analysis

Data were collected from repeat experiments and are presented as means $\pm$ standard deviation (SD). Comparisons between groups were performed by one and two-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA). Results were considered statistically significant with $p < 0.05$. Information on the numbers of animals used in each treatment group and experiment can be found in Suppl. Table 1.

Results

Effect of diet and arsenic on the mouse weight

The body weight and kidney:body weight ratios at 24 weeks are summarized in Table. 1. For the HFD group, there were significant weight gains for both sexes, compared to the LFD-fed group. Arsenic caused a significant weight gain in female mice in the HFD group, but did not affect the body weight in males. There were no significant differences in the kidney:body weight ratios among any of the treatment groups.

Effect of diet and arsenic on kidney histopathology

General morphology—General morphological changes in the kidney were examined following PAS staining (Fig. 1). In both male and female mice, the percent mesangial matrix of HFD fed animals significantly increased relative to that observed in LFD mice at both 10 and 24 weeks. Similarly, for both genders, arsenic treatment increased the percent mesangial matrix. In female mice, arsenic also increased the mesangial matrix accumulation by HFD, compare to LFD/As mice (Fig. 1).

Glomerular area was not affected in any treatment group at 10 weeks (Fig. 1). At 24 weeks, HFD/As-treatment significantly increased glomerular area in both genders, compared to LFD- and LFD/As-treated animals. Additionally, in female mice glomerular area significantly increased in HFD fed animals compared to LFD controls. Following ANOVA, significant gender differences were observed in the 24 week glomerular area ($p<0.02$) and 10 week mesangial matrix ($p<0.01$).

Renal fibrosis—The development of renal fibrosis by arsenic or HFD was analyzed by measuring collagen, fibronectin-1 (FN-1) and connective tissue growth factor (CTGF). In all treatment groups, the degree of collagen deposition, indicated by Picro-Sirius red staining, significantly increased between 10 and 24 weeks (Fig. 2). For male mice at 10 and 24 weeks, arsenic-treatment alone significantly increased collagen deposition compared with the LFD group. At 10 weeks, HFD/As treatment significantly increased collagen deposition compared to HFD alone. In female mice at 10 and 24 weeks, HFD/As-treatments significantly increased collagen levels compared to the LFD group. Additionally, at 10 weeks collagen significantly increased following HFD/As treatment compared to the LFD/As group, while at 24 weeks it increased compared to the HFD group (Fig. 2). No significant gender-specific differences were observed.

The histochemical observations were supported by Western blot analysis of CTGF protein levels (Fig. 2). At 10 weeks, arsenic increased the expression of CTGF and arsenic significantly increased the expression of CTGF induced by HFD. At 24 weeks, the expression of CTGF was significantly increased in the arsenic group compared with LFD group.

Immunohistochemical staining for FN-1 yielded similar results following arsenic or HFD exposure (Fig. 3). First, there was a significant increase in FN-1 levels between 10 and 24 weeks. In both genders, HFD/As treatment caused significant increases in FN-1 levels compared to LFD controls at 10 weeks. In contrast, at 24 weeks all treatment groups showed significant increases in FN-1, compared to LFD mice. Additionally, exposure to arsenic, increased FN-1 deposition in the HFD group. Significant gender-specific differences were observed at 24 weeks.

Similar trends were observed when steady-state FN-1 mRNA levels were measured (Fig 3). Arsenic combined with HFD significantly increased FN-1 mRNA levels in male mice at 10 weeks, compared to LFD controls. In contrast, neither arsenic nor HFD significantly affected female mice. At 24 weeks, FN-1 mRNA levels in male mice significantly increase in arsenic and As/HFD-treated animals, compared to controls. Additionally, arsenic caused a significant increase in FN-1 mRNA levels in HFD fed animals. In female mice at 24 weeks, arsenic and As/HFD caused significant increases in the expression of FN-1 mRNA in the kidney, compared to controls.

Effect of arsenic and HFD on renal inflammation

Changes in the levels of several key inflammatory cytokines; IL-1 β , ICAM-1 and TNF- α ; were determined by Western blot analysis (Fig. 4). In general, male mice showed significant increases in the expression of the inflammatory cytokines in response to arsenic exposure.

High fat diet alone did not affect cytokines expression levels. ICAM-1 levels were not affected after 10 weeks, but at 24 weeks were significantly greater in arsenic and As/HFD-treated mice compared to LFD controls. IL-1 β and TNF- α levels significantly increased in response to arsenic at 10 and 24 weeks.

In contrast, the expression of IL-1 β , ICAM-1 or TNF- α was not significantly affected in female mice at 10 weeks in response to arsenic or HFD. At 24 weeks, there were significant differences in the levels of IL-1 β and TNF- α in LFD/As vs HFD/As mice (Fig. 4). ANOVA confirmed significant gender-specific differences in ICAM-1, IL-1 β and TNF- α responses to arsenic exposure.

The p38 MAPK pathway is a critical regulator of inflammatory cytokines expression and plays an essential role in cell proliferation under environmental stress [23]. Levels of p38 MAPK phosphorylation in response to arsenic and diet there were not significantly affected in male or female mice at 10 weeks (Fig. 4). At 24 weeks however, the level of phospho-p38 in As/HFD-treated male mice was significantly greater compared to LFD control or LFD/As groups, suggesting that arsenic could increase the levels of p38 phosphorylation induced by HFD (Fig. 4). For female mice at 24 weeks, arsenic significantly increased the level of phospho-p38 induced by HFD.

Effect of arsenic and HFD on the oxidative stress response

Immunohistochemical analysis of kidney sections with anti-4-HNE, as an indicator of oxidative stress, showed significant increases in HFD, LFD/As and HFD/As male mice at 10 and 24 weeks, compared to LFD controls (Fig. 5). Additionally, the magnitude of the response significantly increased between 10 and 24 weeks. Similar responses were observed in female mice, with the exception of a lack of response to HFD alone at 10 weeks. ANOVA found significant gender differences in oxidative damage, which could be attributed to the larger response in male versus female mice.

The transcription factor Nrf2 and its downstream targets play important roles in the oxidative stress response [25, 26]. Neither HFD nor arsenic significantly affected Nrf2 levels in female mice (Fig. 6). In male mice at 10 weeks however, arsenic significantly reduced Nrf2 protein levels. Additionally, at 24 weeks both HFD and arsenic reduced Nrf2 levels. Transcriptional activity of Nrf2 was determined by measuring steady-state mRNA levels of antioxidant genes: heme oxygenase 1 (HO1), superoxide dismutase 2 (SOD2) and NAD(P)H quinone dehydrogenase 1 (NQO1) (Table 2). mRNA levels of HO1 and NQO1 were not affected at the 10 week time point under any condition. At 24 weeks, the HFS/As treatment significantly reduced HO1 and NQO1 levels in male and NQO1 in females mice, relative to the LFD controls.

Levels of SOD2 mRNA were not significantly affected in female mice under any condition (Table 2). In male animals however, HFD/As caused a significant reduction in SOD2 mRNA levels at 10 and 24 weeks, compared to LFD control animals. The change in SOD2 mRNA corresponded to changes in Nrf2 and SOD2 protein levels (Fig. 6). Interestingly, in 10 week old female mice, HFD/As lead to a significant increase in SOD2 protein levels, compared to LFD-, LFD/As- and HFD-treated animals.

Discussion

Arsenic intoxication and high fat diet-induced obesity are worldwide epidemics. A two-hit model was developed to examine the effects of whole life exposure to arsenic (hit-one) on the development of HFD-induced (hit-two) renal injury. Additionally, the impact of gender on these responses was examined.

Arsenic alone caused a significant increase in the body weight of female mice, but this response was not observed when combined with HFD (Table 1). In contrast, in male mice arsenic exposure alone did not significantly affect body weight in LFD-fed animals. When combined with HFD; however, there was a greater increase in body weight compared to LFD/As-treated male mice. These effects in mice have not previously been investigated.

Obesity is considered to be a chronic low-grade inflammatory state that can eventually lead to metabolic disorders and kidney disease [27]. High fat diet causes an upregulation of pro-inflammatory cytokines leading to renal hypertrophy and dysfunction [28]. The TNF- α inflammatory pathway can regulate the expression of pro-inflammatory cytokines through the p38 MAPK pathway, which is activated under environmental stress [29]. Increasing evidence supports the importance of the inflammatory cytokines TNF- α , IL-1 β and ICAM-1 in the development of renal insufficiency [30, 31]. The combined exposure to HFD and arsenic significantly increased the phosphorylation of p38 MAPK. This increase was associated with increased levels of inflammatory factors. Additionally, these responses were more prominent in male than female mice (Fig. 4).

The combined exposure of arsenic and HFD significantly increased collagen and fibrosis, and renal pathologies (Figs. 1–3). Additionally, the pathologies were significantly greater in male compared to female mice. Up-regulation of fibrogenic cytokines can lead to the accumulation of extracellular matrix proteins and eventual renal fibrosis [32, 33]. Prolonged exposure to arsenic increases the risk of renal fibrosis by renal epithelial cell epithelial-mesenchymal transition [34]. Both HFD and arsenic significantly increased the expression of fibrosis-related cytokines including CTGF, TNF- α , IL-1 β in mice. These observations are consistent with a model where arsenic and HFD activate an inflammatory response leading to renal tissue remodeling. Furthermore, they suggest that males are more susceptible to HFD- and arsenic-induced renal damage.

Oxidative stress plays a fundamental role in various types of kidney disease [35]. The levels of biomarkers for oxidative stress increase in obese animals, which is associated with reduced antioxidant enzyme activity [36, 37]. Additionally, arsenic induces toxicity via increased levels of oxidative stress and reduced antioxidant defense levels [38]. Arsenic-induced oxidative stress may lead to changes in the expression of antioxidant genes, thereby promoting the production of ROS. This results in an increase in lipid peroxidation and subsequent renal damage. In this study, both arsenic and HFD caused time dependent increases in lipid peroxidation in male and female mice with potentially synergistic effects. The level of oxidative stress-induced injury was greater in male mice compared to females. Several studies have reported sex-specific differences in renal responses to environmentally-

induced oxidative stress in mice. These differences may be a consequence of gender-related differences in the activities of oxidative stress response enzymes [39–41].

After 10 weeks of HFD and arsenic exposure, there was a decrease in Nrf2 protein levels with a corresponding decrease SOD2 protein. mRNA levels of several antioxidant enzymes in HFD/As-treated male mice were reduced, with no significant change in female mice (Table 2). These observations may explain why early renal lesions were not detected in female mice and why HFD and arsenic exposure lead to greater damage in males. These observations are consistent with a study in Taiwan that reported a higher association between the incidence of chronic kidney disease in humans and exposures to > 50 ppb arsenic contaminated drinking water [42].

The results from the current study support a two-hit model where arsenic-induced kidney damage increases due to HFD-induced obesity. Current data support a model where HFD and arsenic increase renal oxidative stress and inflammatory responses leading to structural remodeling of the kidney (i.e., fibrosis). Furthermore, there are gender-specific differences, with males being more susceptible than females. The gender differences in the response may be a result of an increased arsenic methylation efficiency and excretion of arsenicals observed in females [43, 44]. The precise mechanism for this gender difference needs to be further explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Effects of Diet and Arsenic on Glomeruli (Mean ± SD)

Gender	Exposure time	LFD	HFD	LFD/As	HFD/As
Mesangial matrix area (%)					
Male	10 weeks	5.23±0.79	9.61±2.62 ^a	9.06±1.47 ^a	11.55±2.21 ^a
	24 weeks	7.98±1.45	13.71±1.61 ^a	14.26±0.83 ^a	15.07±2.40 ^a
Female	10 weeks	4.89±0.68	9.40±1.23 ^a	7.16±0.61 ^{a,c}	9.50±1.65 ^{a,b}
	24 weeks	6.39±0.74	13.70±1.62 ^a	11.49±3.41 ^a	14.60±3.26 ^a
Glomerular area (μm ²)					
Male	10 weeks	3565±454	3898±396	3427±264	3778±339
	24 weeks	3959±290	4892±374	4105±869	5535±619 ^{a,b}
Female	10 weeks	3319±189	3630±278	3690±450	3824±269
	24 weeks	3329±213	4859±850 ^b	3231±316 ^c	4980±1039 ^{a,b}

^a p<0.05 vs LFD
^b p<0.05 vs LFD/As
^c p<0.05 vs HFD

Figure 1. Effects of arsenic and HFD on pathological changes in the kidney. Representative 40x images of PAS stained of kidney tissue from the four treatment groups (LFD; low fat diet, HFD; high fat diet). The table presents the quantitative analysis of percent mesangial matrix area and glomerular area from PAS stained tissue. Data are presented as means ± SD. Scale bar = 50 μm.

Effects of Diet and Arsenic on Renal Collagen (Mean $\pm$ SD)

Gender	Exposure time	LFD	HFD	LFD/As	HFD/As
Male	10 weeks	0.077 $\pm$ 0.034	0.155 $\pm$ 0.052 ^a	0.198 $\pm$ 0.033 ^a	0.227 $\pm$ 0.039 ^{a,c}
	24 weeks	0.206 $\pm$ 0.070	0.303 $\pm$ 0.054	0.383 $\pm$ 0.080 ^a	0.434 $\pm$ 0.094 ^a
Female	10 weeks	0.100 $\pm$ 0.06	0.170 $\pm$ 0.045	0.138 $\pm$ 0.033	0.220 $\pm$ 0.047 ^{a,b}
	24 weeks	0.205 $\pm$ 0.100	0.239 $\pm$ 0.044	0.363 $\pm$ 0.043	0.454 $\pm$ 0.106 ^{a,c}

^a p<0.05 vs LFD
^b p<0.05 vs LFD/As
^c p<0.05 vs HFD

Figure 2. Effects of arsenic and HFD on collagen expression.

Upper panel, Representative 40x images of collagen stained kidney tissue (LFD; low fat diet, HFD; high fat diet). The table presents the quantitative analysis of collagen levels from Picro-Sirius red stained tissue. Lower panel, fold change of connective tissue growth factor (CTGF) protein levels, relative to β -actin. Data are presented as means $\pm$ SD and are normalized to the CTGF/ β -actin ratio calculated in LFD-treated animals. Horizontal bars represent statistically significant differences ($p<0.05$) between the two groups. Scale bar = 50 μ m.

Effects of Diet and Arsenic on Renal Fibronectin-1 (Mean $\pm$ SD)

Gender	Exposure time	LFD	HFD	LFD/As	HFD/As
Male	10 weeks	0.206 $\pm$ 0.070	0.303 $\pm$ 0.054	0.303 $\pm$ 0.070	0.434 $\pm$ 0.094 ^a
	24 weeks	1.00 $\pm$ 0.44	2.026 $\pm$ 0.675 ^a	2.269 $\pm$ 0.253 ^a	2.960 $\pm$ 0.510 ^{a,c}
Female	10 weeks	0.205 $\pm$ 0.100	0.339 $\pm$ 0.081	0.318 $\pm$ 0.061	0.379 $\pm$ 0.075 ^a
	24 weeks	1.00 $\pm$ 0.60	1.699 $\pm$ 0.315 ^a	1.808 $\pm$ 0.262 ^a	1.870 $\pm$ 0.302 ^a

^a p<0.05 vs LFD^b p<0.05 vs LFD/As^c p<0.05 vs HFD **Figure 3. Effects of arsenic and HFD on fibronectin expression.**

Upper panel, Representative 40x images of fibronectin stained kidney tissue. The table presents the quantitative analysis of fibronectin levels from immunohistochemically stained tissue using anti-fibronectin-1 antibodies. Lower panel, fold change in steady-state FN-1 mRNA levels (light gray: LFD; low fat diet, dark gray: HFD; high fat diet, hatched bars 100 ppb arsenic). Data are presented as means $\pm$ SD and are normalized to FN-1 mRNA levels calculated in LFD-treated animals. Horizontal bars represent statistically significant differences ($p<0.05$) between the two groups.. Scale bar = 50 μ m.

Figure 4. Effects of arsenic and HFD on renal inflammation.

Protein levels of ICAM-1, IL-1β, TNF-α p38 MAPK and phospho-p38 MAPK (P-p38) were measured by Western blot analyses. Data are presented as means $\pm$ SD and are expressed as target protein/ β -actin ratio, except for phospho-p38 MAPK which is expressed and the ratio of the phospho to the non-phospho form. Data are normalized to levels calculated in LFD-treated animals (light gray bars: LFD; low fat diet, dark gray bars: HFD; high fat diet, hatched bars 100 ppb arsenic). Horizontal bars represent statistically significant differences ($p < 0.05$) between the two groups.

Effects of Diet and Arsenic on 4-Hydroxynonenal (Mean $\pm$ SD)

Gender	Exposure time	LFD	HFD	LFD/As	HFD/As
Male	10 weeks	1.09 $\pm$ 0.09	1.91 $\pm$ 0.31 ^a	2.13 $\pm$ 0.08 ^a	2.96 $\pm$ 0.31 ^{a, b, c}
	24 weeks	2.13 $\pm$ 0.15	4.04 $\pm$ 0.55 ^a	3.79 $\pm$ 0.34 ^a	4.81 $\pm$ 0.91 ^a
Female	10 weeks	1.15 $\pm$ 0.17	1.52 $\pm$ 0.14	1.87 $\pm$ 0.26 ^a	2.50 $\pm$ 0.18 ^{a, b, c}
	24 weeks	1.67 $\pm$ 0.37	2.91 $\pm$ 0.18 ^a	3.18 $\pm$ 0.55 ^a	4.69 $\pm$ 0.40 ^{a, b, c}

^a p<0.05 vs LFD

^b p<0.05 vs LFD/As

^c p<0.05 vs HFD

Figure 5. Effects of arsenic and HFD on lipid peroxidation.

Upper panel, Representative 40x images of 4-hydroxynonenal immuno-stained kidney tissue (LFD; low fat diet, HFD; high fat diet). The table presents the quantitative analysis of 4-HNE stained area. Data are presented as means $\pm$ SD.. Scale bar = 50 μ m.

Figure 6. Effect of arsenic and HFD on protein levels of Nrf-2 and superoxide dismutase 2. Protein levels of Nrf-2 and SOD2 were evaluated by Western blot analysis. Data are presented as means $\pm$ SD and are expressed as target protein/ β -actin ratio and normalized to levels calculated in LFD-treated animals (light gray bars: LFD; low fat diet, dark gray bars: HFD; high fat diet, hatched bars 100 ppb arsenic). Horizontal bars represent statistically significant differences ($p < 0.05$) between the two groups.

Table 1.Effects of Arsenic and diet on animal weight (mean $\pm$ SD)

	Gender	LFD	LFD/As	HFD	HFD/As
Body Weight (g)	Male	32.04 $\pm$ 3.18	33.03 $\pm$ 3.67	43.42 $\pm$ 6.30 ^b	42.88 $\pm$ 5.13 ^b
	Female	21.97 $\pm$ 1.19	22.28 $\pm$ 1.82	25.73 $\pm$ 1.78 ^b	29.20 $\pm$ 3.10 ^{a,b}
Kidney: Body Weight (x100)	Male	1.07 $\pm$ 0.25	0.96 $\pm$ 0.30	0.80 $\pm$ 0.16	0.78 $\pm$ 0.03
	Female	1.05 $\pm$ 0.06	1.00 $\pm$ 0.16	1.13 $\pm$ 0.23	0.99 $\pm$ 0.18

^aSignificantly different from LFD/As.^bSignificantly different from LFD.

Table 2Effects of Diet and Arsenic on mRNA Levels of Oxidative Stress Response Genes (Mean $\pm$ SD)

Gender	Exposure time	LFD	HFD	LFD/As	HFD/As
Heme Oxygenase 1					
Male	10 weeks	1.00 $\pm$ 0.12	0.97 $\pm$ 0.10	0.86 $\pm$ 0.20	0.84 $\pm$ 0.11
	24 weeks	1.00 $\pm$ 0.17	0.81 $\pm$ 0.06	0.69 $\pm$ 0.15	0.66 $\pm$ 0.05 ^a
Female	10 weeks	1.00 $\pm$ 0.04	1.18 $\pm$ 0.09	1.06 $\pm$ 0.28	1.19 $\pm$ 0.30
	24 weeks	1.00 $\pm$ 0.05	0.89 $\pm$ 0.13	0.75 $\pm$ 0.10	0.74 $\pm$ 0.09 ^a
NAD(P)H Quinone Dehydrogenase 1					
Male	10 weeks	1.00 $\pm$ 0.15	0.93 $\pm$ 0.21	0.85 $\pm$ 0.14	0.84 $\pm$ 0.06
	24 weeks	1.00 $\pm$ 0.05	0.85 $\pm$ 0.07	0.85 $\pm$ 0.13	0.76 $\pm$ 0.08 ^a
Female	10 weeks	1.00 $\pm$ 0.20	0.98 $\pm$ 0.28	0.93 $\pm$ 0.20	1.10 $\pm$ 0.18
	24 weeks	1.00 $\pm$ 0.07	0.90 $\pm$ 0.07	0.87 $\pm$ 0.11	0.83 $\pm$ 0.16
Superoxide Dismutase 2					
Male	10 weeks	1.00 $\pm$ 0.12	0.90 $\pm$ 0.40	0.86 $\pm$ 0.14	0.69 $\pm$ 0.08 ^a
	24 weeks	1.00 $\pm$ 0.19	0.86 $\pm$ 0.06	0.88 $\pm$ 0.18	0.71 $\pm$ 0.10
Female	10 weeks	1.00 $\pm$ 0.28	0.86 $\pm$ 0.10	1.13 $\pm$ 0.33	1.18 $\pm$ 0.28
	24 weeks	1.00 $\pm$ 0.12	0.93 $\pm$ 0.13	0.92 $\pm$ 0.10	0.77 $\pm$ 0.05

^a p<0.05 vs LFD