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Chronic subhepatotoxic exposure to arsenic enhances hepatic injury caused by high fat diet in mice

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Abstract

Arsenic is a ubiquitous contaminant in drinking water. Whereas arsenic can be directly hepatotoxic, the concentrations/doses required are generally higher than present in the US water supply. However, physiological/biochemical changes that are alone pathologically inert can enhance the hepatotoxic response to a subsequent stimulus. Such a '2-hit' paradigm is best exemplified in chronic fatty liver diseases. Here, the hypothesis that low arsenic exposure sensitizes liver to hepatotoxicity in a mouse model of non-alcoholic fatty liver disease was tested. Accordingly, male C57Bl/6J mice were exposed to low fat diet (LFD; 13% calories as fat) or high fat diet (HFD; 42% calories as fat) and tap water or arsenic (4.9 ppm as sodium arsenite) for ten weeks. Biochemical and histologic indices of liver damage were determined. High fat diet ($\pm$ arsenic) significantly increased body weight gain in mice compared with low-fat controls. HFD significantly increased liver to body weight ratios; this variable was unaffected by arsenic exposure. HFD caused steatohepatitis, as indicated by histological assessment and by increases in plasma ALT and AST. Although arsenic exposure had no effect on indices of liver damage in LFD-fed animals, it significantly increased the liver damage caused by HFD. This effect of arsenic correlated with enhanced inflammation and fibrin extracellular matrix (ECM) deposition. These data indicate that subhepatotoxic arsenic exposure enhances the toxicity of HFD. These results also suggest that arsenic exposure might be a risk factor for the development of fatty liver disease in human populations.

Keywords

sodium arsenite; non-alcoholic fatty liver disease; metabolic syndrome; obesity

Inorganic arsenic is a ubiquitous element and a natural drinking water contaminant (National Research Council, 1999; 2001). Owing to its toxic potential to humans, it is a high priority

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hazardous substance in the United States. Chronic exposure to arsenic has been linked with a myriad of possible health effects, including skin lesions, hypertension, cardiovascular disease, respiratory disease, and malignancies of the skin and internal organs (Waalkes *et al.*, 2004). The liver is a well-known target organ of arsenic exposure. Hepatic abnormalities caused by arsenic exposure include hepatomegaly, non-cirrhotic portal fibrosis and portal hypertension (Santra *et al.*, 1999; 2000; Mazumder, 2005). Furthermore, arsenic exposure has been linked to hepatic malignancies, namely hepatic angiosarcoma and hepatocellular carcinoma in both humans and in animal models (Smith *et al.*, 1992; Waalkes *et al.*, 2006). Straub *et al.* (2007) demonstrated that mouse liver is also sensitive to more subtle hepatic changes (e.g., hepatic endothelial cell capillarization and vessel remodeling) at lower arsenic exposure levels (250 ppb) without any gross pathologic effects. It is nevertheless unclear at this time if environmental arsenic exposure at the levels observed in the US causes liver disease.

Another major health concern for the US population is obesity, the prevalence of which is increasing at an alarming rate (Flegal *et al.*, 1998; Ogden *et al.*, 2006). Among the myriad of health complications associated with obesity (e.g., diabetes, cardiovascular risk, etc.) is non-alcoholic fatty liver disease (NAFLD). The moniker “non-alcoholic” is derived from the fact that the pathology of NAFLD is indistinguishable from that of alcoholic fatty liver disease, but occurs in the absence of significant alcohol consumption (Ludwig *et al.*, 1980). NAFLD is a spectrum of liver diseases, ranging from simple steatosis, to active inflammation, to advanced fibrosis and cirrhosis (Day, 2006). Risk factors for primary NAFLD (i.e., not secondary to other proximate causes) are analogous to those of metabolic syndrome (e.g., obesity, type II diabetes, and dyslipidemia) (Clark, 2006). It is however also clear that there are likely other unidentified risk factors that contribute to the development of disease.

A striking feature of arsenic exposure and non-alcoholic fatty liver disease is that there is significant overlap between areas of risk in the US (Welch *et al.*, 2000; Mokdad *et al.*, 2003). For example, states with clusters of municipal wells with high levels of arsenic [e.g., Michigan, Texas, West Virginia, and Oklahoma (United States Geological Survey, 2007)] also have high incidences of obesity and diabetes (Centers for Disease Control and Prevention, 2007). Furthermore, high arsenic in the drinking water is generally localized to private artesian water supplies (not regulated by the EPA) in rural communities, where the incidence of obesity tends to be even higher than in most areas of the country (National Center for Health Statistics, 2001). It is therefore possible that arsenic exposure is an unidentified environmental risk factor in the development of NAFLD. The purpose of the current study was to test the hypothesis that low dose arsenic exposure sensitizes liver to hepatotoxicity in a mouse model of NAFLD.

Methods

Animals and Treatments

Four week old male C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the local Institutional Animal Care and Use Committee. Food and tap water were allowed ad libitum. Mice were fed AIN-76A Purified diet (Harlan Laboratories, Madison, WI) for one week to reduce potential confounding factors of arsenic present in standard laboratory chow (Kozul *et al.*, 2008). Mice were exposed to sodium arsenite (4.9 ppm in drinking water) or tap water for one week prior to initiating feeding with either low fat diet (13% fat in calories) or high fat diet (42% fat in calories) (Harlan Laboratories, Madison, WI) for 10 weeks. This exposure level of arsenic was determined by preliminary range-finding experiments to cause no overt liver damage in mice fed low-fat diet. Food and

water consumption were measured twice a week. Body weight was measured once a week. For sacrifice, mice were anesthetized with ketamine/xylazine (100/15 mg/kg i.m.). Blood was collected from the vena cava just prior to sacrifice by exsanguination, and citrated plasma was stored at -80°C for further analysis. Portions of liver tissue were frozen immediately in liquid nitrogen, while others were fixed in 10% neutral buffered formalin or embedded in frozen specimen medium (Tissue-Tek OCT compound, Sakura Finetek, Torrance, CA) for subsequent sectioning and mounting on microscope slides.

Clinical analyses and histology

Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), cholesterol (CHOL), albumin and total protein were determined using standard kits (Thermo Scientific, Middletown, VA). Plasma levels of high density lipoprotein (HDL) and low density lipoprotein (LDL) were determined using kits (Wako Chemicals USA, Richmond, VA). Hepatic lipids were extracted and determined as previously described (Bergheim *et al.*, 2006; Kaiser *et al.*, 2009). Plasma insulin (fed) was quantified using an ELISA kit purchased from ALPCO Diagnostics (Windham, NH) (Beier *et al.*, 2008). Plasma levels of glucose (fed) were determined using a glucose assay kit (BioAssay Systems, Hayward, CA) (Beier *et al.*, 2008).

Immunostaining

Formalin-fixed, paraffin-embedded sections (5 μm) were deparaffinized and then rehydrated with graded ethanol solutions. Slides were incubated in primary antibody for F4/80 cell surface receptor (Abcam plc, Cambridge, MA). A secondary antibody, biotinylated anti-Rat IgG was used (Vector Laboratories, Inc., Burlingame, CA). Immunohistochemistry was visualized using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) and Metamorph software. Immunofluorescent detection of fibrin deposition was carried out as described previously (Beier *et al.*, 2009).

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted from liver tissue samples by a guanidium thiocyanate-based method (Tel-Test, Austin, TX). RNA concentrations were determined spectrophotometrically, and 1 μg total RNA was reverse transcribed using a kit (Quanta Biosciences, Gaithersburg, MD). PCR was performed using the ABI StepOne Plus Software. The comparative C_T method was used to determine fold differences between samples. The comparative C_T method determines the amount of target, normalized to an endogenous reference (β -actin) and relative to a calibrator ($2^{-\Delta\Delta C_T}$).

Statistical analyses

Results are reported as means $\pm$ SE ($n = 6-10$). ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) for the determination of statistical significance among treatment groups, as appropriate. A p value less than 0.05 was selected before the study as the level of significance.

Results

Effect of HFD and arsenic on body and organ weight

All animals gained weight during the course of the study and there was no mortality or morbidity in any group during the course of the study. Mice fed LFD+tap water consumed an average of 3.0 ± 0.1 g/d over the course of the study. Mice fed HFD+tap water consumed ~25% more food, with values of 3.8 ± 0.2 g/d. Arsenic exposure did not significantly affect food consumption in either diet group, with values of 2.9 ± 0.1 g/d and 3.4 ± 0.2 g/d, for LFD

and HFD, respectively. Mice fed HFD also gained weight at a significantly more rapid rate (Figure 1A); differences in final average weights between HFD and LFD were ~10 g; arsenic did not significantly alter the effect of HFD on this variable. This increase in body weight caused by HFD was accompanied by a corresponding increase in fat deposition, as indicated by a ~50% increase in the weight of the epididymal fat pads at sacrifice (Figure 1B). Likewise, HFD feeding caused hepatomegaly, as indicated by the almost doubling of liver size in the HFD groups (Figure 1C). The effect of HFD on fat deposition (Figure 1B) and liver size (Figure 1C) and was unaltered by arsenic exposure.

Arsenic exposure enhanced HFD-induced liver injury

Previous studies have shown that feeding mice a diet enriched in triglycerides and cholesterol (i.e., 'Western diet') causes obesity, insulin resistance and fatty liver injury [e.g., (Wouters *et al.*, 2008)], with pathology similar to that found in human non-alcoholic fatty liver disease (NAFLD). As expected by the hepatomegaly observed (Figure 1C), 10 weeks of HFD feeding dramatically increased lipid accumulation in the liver (steatosis; Figure 2A, lower left panel); this pathologic change comprised both macrovesicular and microvesicular steatosis. HFD feeding also increased the appearance of necroinflammatory foci in the liver. This dietary exposure significantly increased plasma activity of alanine aminotransferase (ALT) ~5-fold. Exposure to arsenic did not significantly affect the macroscopic histology or plasma transaminase values in LFD-fed mice. There were no apparent gross morphologic changes in livers from HFD+arsenic (Figure 2A, lower right panel) in comparison to the HFD+tap group. In contrast, both ALT and AST activities in the plasma of HFD+arsenic were significantly higher than those exposed to tap (Figure 2B), indicative of more liver injury.

Arsenic exposure did not alter changes in plasma insulin or glucose, or fatty liver caused by HFD feeding

Feeding HFD to rodents or humans is known to increase blood glucose, which is indicative of impaired insulin signaling. Arsenic exposure had no significant effect on plasma glucose or insulin in LFD-fed mice (Table 1). HFD feeding in tap-exposed animals increased plasma glucose levels significantly, although insulin levels remained the same (Table 1); the addition of arsenic to the drinking water did not significantly alter plasma glucose or insulin compared to the HFD+tap group. As expected from the histologic changes (see Figure 2A), HFD feeding significantly increased the levels of FFA (~1.4-fold), TG (~2-fold), and cholesterol (~3-fold) in the liver (Figure 3). Hepatic content of phospholipids was not altered by high fat diet feeding. Arsenic exposure did not significantly change the effect of HFD on any of these variables. Plasma levels of cholesterol, HDL and LDL were also significantly increased by HFD feeding (Table 1). None of these variables were significantly affected by arsenic coexposure.

Effects of high fat diet and arsenic on hepatic inflammation

Previous work by this group showed that arsenic preexposure enhances the hepatic inflammatory response to bacterial LPS (Arteel *et al.*, 2008). The exacerbated liver damage caused by arsenic coexposure to HFD-fed mice could also be mediated by elevated inflammation. Therefore, the effect of HFD and arsenic on indices of hepatic inflammation were determined (Figure 4). Ten weeks of HFD significantly increased hepatic mRNA expression of plasminogen activator inhibitor-1 (PAI-1; ~3-fold), IL-6 (~3-fold) and IL-10 (~2.5-fold) (Figure 4A). Arsenic exposure had no effect on expression of any of these genes in mice fed LFD. In mice fed with HFD, arsenic exposure significantly increased TNF α expression, and enhanced the effect of HFD on PAI-1 expression. The increase in IL-10 expression caused by HFD was significantly attenuated by arsenic coexposure. The expression of IL-6 was unaffected by arsenic exposure. Although it had no effect on this

variable in the LFD group, arsenic coexposure significantly increased the number of F4/80-positive cells in the HFD group (Figure 4B).

PAI-1 is a major inhibitor of both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), and is therefore a key regulator of fibrin degradation (i.e., fibrinolysis) by plasmin (Kruithof, 1988). The effect of HFD and arsenic exposure on fibrin extracellular matrix (ECM) accumulation in liver was therefore determined by immunofluorescence (Figure 5). Low-level fibrin ECM was detected in the perisinusoidal areas of livers from LFD+tap mice (Figure 5); arsenic coexposure did not significantly increase the extent of fibrin staining in this dietary group (Figure 5). HFD alone significantly increased fibrin deposition ~4-fold in comparison with LFD controls; arsenic significantly enhanced fibrin staining caused by HFD by ~2-fold.

Arsenic did not enhance hepatic fibrosis

Fibrosis is a common pathologic response to chronic hepatic injury. The effect of HFD feeding and arsenic exposure on indices of hepatic fibrosis and fibrogenesis were therefore determined (Figure 6). HFD feeding for 10 weeks caused perisinusoidal fibrosis in liver, as determined by Sirius red staining (Figure 6A, lower left panel). Coexposure of arsenic in the drinking water did not alter this effect of HFD (Figure 6A, lower right panel). Increases in the expression of α -smooth muscle actin (α SMA), TGF β , and collagen I α 1 are indicative of stellate cell activation and increased ECM synthesis in liver. The effect of HFD and arsenic on hepatic expression of these genes was therefore determined (Figure 6B). HFD diet feeding for 10 weeks significantly increased expression of all 3 of these genes; arsenic coexposure did not significantly affect expression in either LFD- or HFD-fed mice (Figure 6B). The expression of prolyl-4-hydroxylase (α subunit), a component of the enzyme responsible for crosslinking of collagen fibrils, was unaffected by either diet or arsenic under these conditions (Figure 6B, lower right panel).

Discussion

Nearly 4,000 wells providing community water in the U.S. have arsenic levels greater than the current WHO recommended maximum contaminant level (MCL) of 10 μ g/L (Engel *et al.*, 1994; Frost *et al.*, 2003). Furthermore, even higher arsenic concentrations may be found in private artesian water supplies not regulated by the Safe Drinking Water Act. As mentioned in the Introduction, although the liver is a known target organ of chronic arsenic exposure, such effects are observed primarily in regions of the world with arsenic concentrations in the drinking water that are much higher than generally observed in the US [e.g., Bangladesh (Santra *et al.*, 1999)]. Therefore, whether or not the concentrations/doses required to achieve significant hepatotoxicity are relevant to US exposure levels is unclear.

Previous work by this group has shown that arsenic exposure, at concentrations that aren't overtly hepatotoxic, enhances lipopolysaccharide-induced liver injury in mice (Arteel *et al.*, 2008). Numerous studies have now established that physiological/biochemical changes to liver that are pathologically inert can enhance the hepatotoxic response caused by a second agent; this '2-hit' paradigm has been best exemplified in fatty liver diseases (Day *et al.*, 1998). For example, Yang *et al.* (1997) demonstrated that livers from genetically obese (fa/fa) rats are exquisitely sensitive to hepatotoxicity caused by the injection of bacterial lipopolysaccharide (LPS) compared to their lean littermates; this exacerbation of liver damage was characterized by a more robust inflammatory response and enhanced cell death. A similar effect was observed in the response to LPS after arsenic exposure (Arteel *et al.*, 2008). It was therefore hypothesized here that subhepatotoxic arsenic exposure would enhance liver damage caused by high-fat diet in mice, which resembles NAFLD.

Hepatic changes caused by arsenic exposure include altered lipid metabolism, steatosis, enhanced inflammation and fibrosis (Santra et al., 2000; Mazumder, 2005). Similar changes are involved in the development and progression of NAFLD (Choi et al., 2005; Diehl et al., 2005). Furthermore, arsenic may increase the risk of developing type II diabetes in humans (Navas-Acien et al., 2009), which is a known risk factor for NAFLD (Clark, 2006). Indeed, recent work has shown in experimental animals that arsenic enhances insulin resistance caused by HFD in mice (Paul et al., 2011). The effect of arsenic and LPS on indices of these potential mechanisms was therefore determined. Arsenic did not alter hepatic or plasma lipid profiles, neither in the LFD or HFD groups (Figures 2 and 3; Table 1) indicating that the enhancement of HFD-induced liver damage by arsenic is not via increased steatosis. Likewise, arsenic did not alter the increase in plasma fed glucose caused by HFD in mice (Table 1). Lastly, arsenic exposure had no apparent effect on the increase in indices of fibrosis caused by HFD in this study (Figure 6). In contrast to steatosis, glucose levels and fibrosis, arsenic exposure did enhance increases in indices of inflammation caused by HFD (Figure 4). Specifically, arsenic increased the expression of the pro-inflammatory cytokine TNF α in the HFD group. Furthermore, arsenic blunted the increase in expression of the anti-inflammatory cytokine, IL-10 in the HFD group. It is proposed that the imbalance between pro- and anti-inflammatory cytokines is a critical step in NAFLD (Tilg, 2010). Arsenic also increased the number of activated macrophages, as determined by F4/80 staining (Figure 4B). Taken together, these results suggest that arsenic enhanced liver damage caused by HFD by increasing the inflammatory response.

Arsenic also significantly enhanced expression of plasminogen activator inhibitor-1 (PAI-1) in HFD-fed mice. This effect correlated with an increase in perisinusoidal deposition of fibrin ECM in the liver (Figure 5). Previous work by this group and others have shown a correlation between fibrin ECM and inflammation in models of hepatic injury [e.g., (Bergheim et al., 2005; 2006; Beier et al., 2009)]. There are several potential mechanisms by which fibrin ECM is proinflammatory. For example, fibrin clots disrupt the flow of blood within the hepatic parenchyma (i.e., hemostasis); the subsequent microregional hypoxia and hepatocellular death may directly and indirectly increase a proinflammatory response (Hewett et al., 1995; Wanless et al., 1995; Pearson et al., 1996; Ganey et al., 2004). Furthermore, fibrin matrices have been shown to be permissive to chemotaxis and activation of monocytes and leukocytes (Holdsworth et al., 1979; Loike et al., 1995). Therefore, the increase in PAI-1 caused by arsenic exposure in the HFD group could contribute to hepatic inflammation by enhancing fibrin ECM deposition.

There are significant differences between the absorption and elimination of arsenic between rodents and humans, making direct comparison of exposure levels difficult between the species; for example, exposure of rodents to 50 ppm arsenic resulted in plasma levels that were only ~10-fold higher than humans consuming 50–100 ppb (Hall et al., 2006). Nevertheless, the exposure level (4.9 ppm) of arsenic used in this study, while in the range of published rodent studies [e.g., (Santra et al., 2000; Chen et al., 2004)], is still high relative to human exposure in the US (ppb). Furthermore, although arsenic appeared to enhance liver damage caused by HFD under these conditions, too few concentrations were employed to determine the nature of this potential interaction (e.g., additive, synergistic, etc). Therefore, although these results serve as proof-of-concept of the potential interaction between arsenic and other hepatotoxicants, future studies are required to validate these findings with lower exposures of arsenic in more chronic models of liver diseases.

Summary and Conclusions

There are many gaps in our understanding of the relative safety of arsenic to the human population. Importantly, most studies to date have focused on the effect of arsenic alone and

not taken into consideration risk-modifying factors. Furthermore, even fewer studies have tested the possibility that arsenic may be a risk modifying factor for other diseases. It was shown here that arsenic enhances experimental NAFLD in mice. Although the increase in liver injury caused by arsenic under these conditions, NAFLD is a chronic disease that requires several years to progress (Adams *et al.*, 2005), so relatively small changes can be greatly amplified over time. These results therefore suggest that the relative risk of hepatic damage caused by arsenic exposure may have to be modified to take into account other mitigating factors, such as underlying fatty liver disease.

Highlights

- Characterizes a mouse model of arsenic enhanced NAFLD.
- Arsenic synergistically enhances experimental fatty liver disease at concentrations that cause no overt hepatotoxicity alone
- This effect is associated with increased inflammation

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A **B**

Figure 1. Effect of high fat diet on growth and organ weight

Body weight over time (A), epididymal fat pad weight (B) and liver weight (C) for tap water or arsenic exposed mice fed with low fat or high fat diet for 10 weeks are shown. Data are means $\pm$ SEM ($n = 6-10$). ^a, $p < 0.05$ compared to low fat diet.

Figure 2. Effect of high fat diet and arsenic on liver injury

A: Representative photomicrographs (200×) depicting hematoxylin & eosin (H&E) stains are shown. B: Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in tap water or arsenic exposed mice after 10 weeks of diet feeding are shown. Quantitative data are means $\pm$ SE (n = 6–10). ^a, $p < 0.05$ compared to low fat diet. ^b, $p < 0.05$ compared to tap.

Hepatic lipid level

Figure 3. Effect of high fat diet and arsenic on hepatic lipids

Hepatic levels of free fatty acids (FFA), triglycerides (TG), cholesterol (CHOL) and phospholipids (PPL) in mice exposed to tap water or arsenic water after 10 weeks of low fat or high fat feeding are shown. Data are means $\pm$ SEM ($n = 6-10$). ^a, $p < 0.05$ compared to low fat diet.

Figure 4. Effect of high fat diet and arsenic on hepatic inflammation

A: Effect of high fat diet and arsenic on hepatic mRNA expressions of tissue necrosis factor- α (TNF α), plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6) and interleukin-10 (IL-10) are shown. Real-time RT-PCR was performed as described in *Methods*. B: Representative photomicrographs (200 $\times$; upper panel) and quantitation (lower panel) of F4/80 immunostains are shown. Quantitative data are means $\pm$ SE ($n = 6-10$) and are expressed as fold of control. ^a, $p < 0.05$ compared to low fat diet. ^b, $p < 0.05$ compared to tap.

Figure 5. Effect of high fat diet and arsenic on fibrin deposition

Representative photomicrographs (200 $\times$; upper panel) and quantitation (lower panel) of fibrin immunofluorescence (green color) are shown. Quantitative data are means $\pm$ SE ($n = 6-10$) and are expressed as fold of control. ^a, $p < 0.05$ compared to low fat diet. ^b, $p < 0.05$ compared to tap.

Figure 6. Effect of high fat diet and arsenic on hepatic fibrosis

A: Representative photomicrographs (200 $\times$) of Sirius Red stains with 400 $\times$ insets are shown. B: Effects of high fat diet and arsenic on hepatic mRNA expressions of alpha-smooth muscle actin (α SMA), transforming growth factor beta (TGF β), Collagen1a1 and prolyl-4-hydroxylase (P4H) are shown. Real-time RT-PCR was performed as described in *Methods*. Quantitative data are means $\pm$ SE ($n = 6-10$) and are expressed as fold of control. ^a, $p < 0.05$ compared to low fat diet. ^b, $p < 0.05$ compared to tap.

Table 1
Effect of high fat diet and arsenic on plasma profiles

Animals were fed with low fat or high fat diet for 10 weeks with either tap or arsenic water as described in *Methods*. Plasma levels of glucose, insulin, lipids, albumin and total protein were measured.

	Plasma profile			
	Low fat diet		High fat diet	
	tap water	arsenic water	tap water	arsenic water
Glucose (mg/dl)	315 ± 31	349 ± 23	520 ± 45 ^a	460 ± 44 ^a
Insulin (ng/dl)	68 ± 19	52 ± 11	63 ± 10	68 ± 15
Cholesterol (mg/dl)	106 ± 4.	115 ± 8	222 ± 21 ^a	180 ± 8 ^a
Triglycerides (mg/dl)	31 ± 2	36 ± 3	38 ± 6	28 ± 6
HDL (mg/dl)	97 ± 4	91 ± 5	121 ± 6 ^a	134 ± 8 ^a
LDL (mg/dl)	15 ± 2	16 ± 3	27 ± 3 ^a	30 ± 2 ^a
Albumin (g/dl)	2.2 ± 0.1	2.3 ± 0.2	2.4 ± 0.1	2.3 ± 0.1
Total protein (g/dl)	3.4 ± 0.1	3.4 ± 0.3	3.9 ± 0.2	3.7 ± 0.1

Data are mean ± SE (n=5–8).

^a $p < 0.05$ compared to low fat diet.

Abbreviations: high density lipoprotein (HDL), low density lipoprotein (LDL).