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Selenium Supplementation Decreases Hepatic Fibrosis in Mice After Chronic Carbon Tetrachloride Administration

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Abstract

Oxidative stress stimulates fibrogenesis, and selenium (Se) has antioxidant properties. This study determined whether Se supplementation affects CCl₄-induced liver injury and fibrosis. Mice were administered CCl₄ over 4 weeks, while controls received olive oil. Se was provided as sodium selenite in the drinking water. Se increased liver Se-dependent glutathione peroxidase activity and decreased liver malondialdehyde after CCl₄. Se decreased liver inflammation but not necrosis caused by CCl₄. Se increased hepatocyte apoptosis after CCl₄ and the pro-apoptotic BAX and Bcl Xs/l proteins. Stellate cell apoptosis occurred only after CCl₄ in Se-supplemented mice. Se decreased stellate cell number and fibrosis after CCl₄. Liver matrix metalloproteinase-9 increased after CCl₄ with Se supplementation. In conclusion, Se supplementation decreased hepatic fibrosis after CCl₄ in the setting of decreased inflammation but increased apoptosis. The principal mechanisms for the decreased fibrosis are a lower number of collagen-producing stellate cells and increased collagen degradation.

Keywords

Selenium; Stellate cells; Collagen; Metalloproteinases; Caspases; Apoptosis

Introduction

Oxidative stress stimulates fibrogenesis [1]. Lipid peroxidation products enhance $\alpha_1(I)$ collagen expression and collagen synthesis by stellate cells in culture [2,3]. Reactive oxygen species (ROS) are mostly generated by increased NAD(P)H oxidase which produces superoxide anion ($O_2^{\cdot-}$) from oxygen (O_2). Superoxide anion in turn generates H_2O_2 and hydroxyl radical ($OH^{\cdot}$). Liver fibrosis is less after chronic CCl_4 administration in mice deficient in the gp91phox subunit of the NADPH complex than in wild-type control mice [4,5].

Selenium is a trace element which has antioxidant properties because it is an essential component of some oxidoreductase enzymes [6]. Most notable is the selenium-dependent glutathione peroxidase enzyme (Se-GSH-PX) which catalyses the reduction of hydrogen peroxide (H_2O_2) to water (H_2O) by conversion of reduced glutathione (GSH) to its oxidized form (GSSG). Selenium and Se-GSH-PX deficiency are associated with higher levels of ROS and lipid peroxidation [6], which can be reversed by Se supplementation [6,7]. Serum and liver Se are decreased in alcoholic and nonalcoholic liver diseases, most likely due to decreased dietary intake [8,9]. In one study, administration of vitamin E together with Se and zinc resulted in a lower 1-month mortality in patients with alcoholic hepatitis [10]. In other studies, however, administration of Se together with vitamins A and E did not affect survival of patients with alcoholic hepatitis [11]. Selenium-enriched lactobacillus decreased liver injury and lipid peroxidation induced by CCl_4 administration in mice [12], while supplementation of the diet with vitamin E and Se decreased hepatic fibrosis produced in rats by acute and chronic CCl_4 administration [13].

The purpose of this study was to determine whether Se supplementation affects liver injury and fibrosis produced by chronic CCl_4 administration in mice.

Materials and Methods

Animals and Materials

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All animals received humane care in compliance with the guidelines from the Animal Care and Use Committee of The Johns Hopkins University. Sirius Red was obtained from Polysciences, Warrington, PA, USA. Carbon tetrachloride (CCl_4), sodium selenite, and goat anti-mouse α -smooth muscle actin Cy3 conjugate antibody were purchased from Sigma Chemical, St. Louis, MO, USA. Anti-mouse thioester *s*-methyltransferase (TEMT) antibody was a gift from Dr. Linda A. Toth from the Southern Illinois University School of Medicine, Springfield, IL, USA. Caspase 3 and caspase 8 fluorometric assay kits were obtained from BioVision (Mountain View, CA, USA).

Animal Treatment

Mice 4–6 weeks of age and weighing 20–30 g were kept in a temperature-controlled room with an alternating 12-h dark and light cycle. Thirty-two mice were divided into four experimental groups of eight mice each as follows: (1) CCl_4 injections; (2) CCl_4 injections with the drinking water supplemented with Se; (3) olive oil injections; and (4) olive oil injections with the drinking water supplemented with Se. CCl_4 was administered as biweekly intraperitoneal (i.p.) injections of 5 μ l of a 20% solution of CCl_4 in olive oil per gram body weight (1.0 ml/kg of CCl_4). Se supplementation was provided as sodium selenite (4 mg of anhydrous sodium selenite/L) in the drinking water. This supplementation was initiated 2 days before starting the CCl_4 or olive oil injections. The water volume ingested was recorded. The animals were on ad lib rodent diet (# 2018X) obtained from Harlan Teklad, Madison, WI, USA, which contains 200 μ g of Se per kilogram of diet. The animals

were killed 4 weeks after the start of these injections. At the time of killing, blood was obtained from the aorta for measurement of serum alanine aminotransferase (ALT), and the samples were stored at -20°C . The liver was removed, rinsed with phosphate-buffered saline (PBS), and divided into five portions: (a) fixed in 10% buffered formaldehyde formalin and embedded in paraffin; (b) snap frozen at -70°C for sectioning and immunohistochemistry; (c) homogenized in appropriate buffer(s) and aliquots frozen at -70°C for biochemical assays; (d) snap frozen at -70°C for Se assay; and (e) placed in RNA STAT-60 (from Tel-Test, Friendswood, TX, USA) solution, homogenized, and stored in -70°C for RNA isolation.

Liver Selenium and Selenium-Dependent Glutathione Peroxidase Activity

Liver Se was determined in liver homogenates by the Oscar E. Olson Biochemistry Laboratories of the South Dakota State University using the Association of Official Analytical Chemists official fluorometric method 996.16 [14]. The activity of Se-dependent glutathione peroxidase was determined in the liver supernatant, obtained after centrifugation of the liver homogenate at $3,000\times g$ for 10 min, with H_2O_2 as a substrate using the glutathione cellular activity assay kit obtained from Sigma-Aldrich, St Louis, MO, USA.

Liver Histology and Morphometric Collagen Determination

The liver sections imbedded in paraffin were cut ($5\mu\text{m}$) and stained with hematoxylin–eosin (H&E), Masson's trichrome, or Sirius red. The extent of necrosis and inflammation was evaluated on blinded slides by M.S.T. from our Department of Pathology. Fibrosis was determined histologically by measuring the intensity of fibrosis in four to six ($\times 100$) digital images captured from slides of each mouse stained with Sirius red. The total fibrosis density score was determined by dividing the image intensity by the image area as described previously [15].

Quantitation of Stellate Cells

Immunofluorescent staining for α -smooth muscle actin (α -SMA) was done in deparaffinized liver sections. The slides were washed in PBS, followed by blocking using PBS–5% fetal bovine serum (FBS). The slides were incubated with Cy3 conjugated monoclonal α -SMA antibody (Sigma, 1:500 in PBS–5% FBS) for 1 h at room temperature and subsequently at 4°C overnight. After washing with PBS four times for 15 min, the slides were mounted and eight areas per slide captured by fluorescent microscopy (magnification $\times 100$). Stellate cells were counted in the eight fields per slide for each mouse.

Serum Alanine Aminotransferase

Serum alanine aminotransferase was determined by the spectrophotometric method of Bergmeyer et al. [16].

Liver Malondialdehyde

Liver slices were homogenized with cold 1.15% KCl, and malondialdehyde was determined using thibarbituric acid by the method of Uchiyama and Mihara [17].

Determination of Messenger RNA by Real-Time Quantitative Polymerase Chain Reaction

The 7900 HT (Applied Biosystems, Foster City, CA, USA) and the SDS 2.2.1 software were used to perform real-time quantitative polymerase chain reaction (RT-qPCR) at The Johns Hopkins DNA Analysis Facility. Total cellular RNA from a portion of liver was placed in RNA STAT 60 reagent, and following their protocol, RNA was purified and isolated. The concentration of the isolated RNA was determined from the optical density at 260 nm and its purity from the 260/280 nm OD ratio. The isolated RNA was stored at -80°C . RT-qPCR for

α_1 (I) collagen mRNA was performed using sequence-specific probes from TaqMan gene expression assays of Applied Biosystems. The probes for mouse α_1 (I) collagen and β -actin (as endogenous control) were obtained from Applied Biosystems. Superscript III first-strand synthesis from Invitrogen (Carlsbad, CA, USA) was used to synthesize first-strand cDNA from the purified RNA. Gene-transcript levels of the above probes were compared to β -actin, the housekeeping endogenous control. Variation in the amount of the transcripts was corrected by the level of expression of the β -actin gene in each individual sample.

Western Blot Analysis

Liver sections were homogenized in 50 mM Tris-HCl buffer pH7.6 containing 150 mM NaCl, 10 mM CaCl_2 , 0.25% Triton-X, 0.1 μM phenylmethanesulfonyl fluoride, 10 μM leupetin, 10 μM pepstatin, 0.1 mM iodoacetamide, and 25 μg aprotinin and then centrifuged at $3,000\times g$ for 10 min at 4°C . The cytosol protein in the supernatant was initially stored at -80°C . The proteins were separated on mini-SDS gels at 100 V for 1 h and electrotransferred to nitrocellulose transblot membranes (BioRad, Hercules, CA). The membranes were washed in PBS, pH7.6, containing 0.1% Tween 20 (PBS-T), blocked with 5% (W/V) dry nonfat milk in PBS-T for 1 h, rinsed with PBS-T, and then incubated with either rabbit anti-mouse antibodies to Fas, Bax, BclXs/l, Bcl-2, cytochrome C, MMP-2, MMP-9, TIMP-1, TIMP-2, or β -actin, obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The membranes were also incubated with rabbit anti-mouse antibody to TEMT, kindly provided by Dr. Linda A Toth. After repeated washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution; Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 h. The membranes were then washed again and visualized by enhanced chemiluminescence reaction (ECL Plus; Amersham Biosciences). Densitometry was determined using Image J v 1.30 obtained from NIH.

Terminal Deoxynucleotidyl Transferase-Mediated Nick-End Labeling Assay

Terminal deoxynucleotidyl transferase-mediated in situ nick-end labeling (TUNEL) assay was performed on paraffin-embedded liver slices with the cell death detection kit from Roche Applied Science (Nutley, NJ, USA). Fluorescence microscope using the fluorescein isothiocyanate filter revealed the apoptotic bodies which were counted.

Apoptosis in Stellate Cells

Apoptosis in stellate cells was determined in 5 μM sections of cryopreserved liver tissue with the cell death detection kit from Roche Applied Science. Following TUNEL staining, the sections were blocked in 5% FBS for 60 min and incubated with Cy3-conjugated monoclonal α -smooth muscle actin antibody (Sigma, 1:500 in PBS-5% fetal bovine serum) for 1 h at room temperature. 4',6-Diamidino-2-phenylindole (DAPI) for nuclear staining was added during the last 10 min of the incubation. The sections were washed in PBS for 1 h with frequent changes. The immunostained slices were evaluated and photographed with laser confocal microscopy.

Caspase Activities

The activities of caspase 3 and caspase 8 were determined in liver homogenates by measuring proteolytic cleavage of the specific fluorogenic substrates, DEVD-7-amino-4-trifluoromethyl coumarin (AFC) and IETD-AFC, respectively (BioVision). The results are expressed as relative units per milligram of protein. The concentration of protein was determined by the method of Lowry et al. [18].

Statistical Analysis

In most measurements, the mean and the standard error of the mean were calculated. The data was analyzed with the Student's *t* test or by two-way analysis of variance when comparing means of more than two groups.

Results

Liver Selenium and Selenium-Dependent Glutathione Peroxidase

The daily ingestion of water containing sodium selenite (4 mg/L) was 9.46 ± 0.53 and 9.24 ± 0.53 ml in mice receiving CCl₄ or olive oil injections, respectively. The calculated mean daily ingestion of the supplement of sodium selenite was 37.8 and 37.0 μg in mice injected with CCl₄ or olive oil, respectively. Liver selenium (Se) was decreased after CCl₄ treatment ($p < 0.01$; Fig. 1). Se supplementation increased liver Se in the control and CCl₄-treated mice ($p < 0.01$), but the enhanced levels of Se were lower in the CCl₄-treated than in the control mice ($p < 0.01$). Liver selenium-dependent glutathione peroxidase enzyme was also lower after CCl₄ treatment ($p < 0.05$; Fig. 1), and in this case, Se supplementation resulted in higher levels of Se-GSH-PX in the control and treated mice. Thioether *s*-methyltransferase, which is important in the detoxification of Se compounds, since it converts dimethylselenide to trimethylselenonium which is water-soluble and excreted in the urine, was decreased by chronic CCl₄ administration regardless of Se supplementation ($p < 0.05$; Fig. 1).

Liver malondialdehyde was not increased significantly by CCl₄ (Fig. 1). Se supplementation decreased liver malondialdehyde in the control ($p < 0.05$) and CCl₄-treated ($p < 0.01$) mice.

Liver Injury and Fibrosis

The morphological changes of liver injury and fibrosis caused by CCl₄ were visualized in sections stained by H&E (not shown) and Sirius red (Fig. 3). The changes include necrosis, inflammation with macrophages, lymphocytes, and fibrosis. Fatty infiltration was minimal. The grades of inflammatory changes were lower after CCl₄ in the Se-supplemented mice ($p < 0.01$; Fig. 2). Se supplementation did not affect the grade of necrosis caused by CCl₄ treatment.

Liver fibrosis was less evident in the Se-supplemented mice (Fig. 3a, b). The area of hepatic fibrosis detected by Sirius red staining and densitometric analysis was significantly lower in the Se-supplemented mice after CCl₄ as compared to the other mouse groups ($p < 0.01$; Fig. 3c). The number of stellate cells, identified by α -smooth muscle actin staining, was lower per $\times 100$ field after CCl₄ in the Se-supplemented mice (Fig. 3d, e). The values were 17.12 ± 1.3 in Se-supplemented as compared to 22.0 ± 1.2 in the control mice ($p < 0.05$).

Serum alanine aminotransferase was elevated to a similar degree after CCl₄ administration in Se-supplemented and control mice (Fig. 2).

The increases in $\alpha_1(I)$ collagen mRNA after CCl₄ administration were similar regardless of whether or not the mice had received Se supplementation ($p < 0.01$; Fig. 4).

Apoptosis

The number of apoptotic hepatocytes was highest after CCl₄ administration in the Se-supplemented mice (Fig. 5a, b). The values from 40–100 fields ($\times 100$) examined were 2.5 ± 0.55 and 4.0 ± 0.70 apoptotic cells per field for the control and Se-supplemented mice, respectively, and 11.5 ± 1.00 and 19.9 ± 1.55 for CCl₄ administration without and with Se supplementation, respectively ($p < 0.01$).

Stellate apoptosis was evaluated by TUNEL assay under confocal microscopy. Apoptosis was detected in a few stellate cells in the livers of mice treated with CCl₄ and supplemented with Se but not in the mice that did not receive Se supplementation (Fig. 6).

Fas (CD95/APO-1) receptor, which mediates apoptosis principally via the extrinsic death receptor pathway, was increased after CCl₄ treatment in the Se-supplemented mice ($p<0.05$; Fig. 7). Activated caspases 3 and 8 are essential in the extrinsic death receptor pathway of apoptosis. Caspase 3 was not changed significantly by chronic CCl₄ administration or Se supplementation (Fig. 7). By contrast, caspase 8 was increased after CCl₄ ($p<0.05$), and this effect was abrogated in the CCl₄ mice receiving Se supplementation ($p<0.05$; Fig. 7).

Components of the mitochondrial pathway of apoptosis that respond to intracellular stress signals were examined. Pro-apoptotic BAX and Bcl Xs/l proteins were only increased after CCl₄ treatment in the Se-supplemented mice ($p<0.05$; Fig. 8). The anti-apoptotic protein Bcl-2 was not changed significantly after CCl₄. Also, cytosolic cytochrome c, which is released from the mitochondria during apoptosis and is regulated by both pro- and anti-apoptotic members of the Bcl-2 family of proteins, was not changed significantly after CCl₄ (Fig. 8).

Matrix Metalloproteinases

Liver matrix metalloproteinase-9 (MMP-9) was only increased after CCl₄ in the Se-supplemented mice ($p<0.05$; Fig. 9), while matrix metalloproteinase 2 (MMP-2) was not changed by CCl₄ in either group of mice (Fig. 9). Tissue inhibitor of metalloproteinase 2 (TIMP-2) was only increased after CCl₄ in the Se-supplemented mice ($p<0.05$; Fig. 9), while TIMP-1 remained unchanged after CCl₄ treatment (Fig. 9).

Discussion

This study shows that dietary selenium (Se) supplementation reduces hepatic fibrosis produced in mice by chronic CCl₄ administration. Hepatic fibrosis after CCl₄ administration was previously shown to be decreased in rats by dietary supplementation with vitamin E and Se [13]. The lesser accumulation of fibrosis in the Se-supplemented mice after CCl₄ administration in our study was associated with a lower number of stellate cells. A combination of decreased formation and increased degradation of collagen was considered as causes of the lesser accumulation of collagen in the liver. The similar increases in $\alpha_1(I)$ collagen mRNA in the Se-supplemented as compared to the control mice after chronic CCl₄ administration indicates that Se does not affect Type I collagen transcription but does not rule out effects of Se on collagen translation. Furthermore, there is evidence in this study that increased collagen degradation contributes to the lesser accumulation of hepatic fibrosis in the Se-supplemented mice. Increased collagen formation from CCl₄ administration [19,20] and from other insults [21] is associated with an increase in collagen degradation. In this study, MMP-9 was increased after CCl₄ in the Se-supplemented mice, while MMP-2 remained unchanged. TIMP-1 also remained unchanged after CCl₄ treatment, while TIMP-2 increased after CCl₄ in the Se-supplemented mice. TIMPs inhibit secretion and activation of metalloproteinases, hence inhibiting collagen degradation [22]. TIMP-2 is not only a specific inhibitor of MMP-2, but it is also required for the activation of MMP-2 [22]; however, in our study, the increase in TIMP-2 was not associated with a change in MMP-2. Of note is that methylselenol increased pro-MMP-2 and TIMP-2 but decreased active MMP-2 in HT1080 tumor cells in culture [23].

The lesser accumulation of collagen after CCl₄ administration in the Se-supplemented mice was associated with less hepatocellular inflammation but similar grades of necrosis and similar elevations of serum ALT than seen in the nonsupplemented mice. Se is known to

have anti-inflammatory properties [24]. Se supplementation was shown to decrease inflammation in lung tissues induced by bacterial endotoxin lipopolysaccharide (LPS) in Se-deficient mice [25]. As regards this study, LPS is known to contribute to CCl₄ hepatotoxicity [26].

Se supplementation increased hepatocyte apoptosis in the mice treated with CCl₄. In a previous study, Se supplementation increased stellate cell apoptosis after acute CCl₄ administration [27]. Large concentrations of sodium selenite (10 μM) induce oxidative stress and apoptosis in cultured HepG₂ cells [27]. This effect of high levels of Se is the result of depletion of GSH by its reaction with the oxidized form of selenium (SeO₄²⁻) [28].

Apoptosis of stellate cells was only found in the CCl₄-treated mice that had received Se supplementation. Iredale et al. [29] showed that apoptosis of stellate cells contributes to resolution of fibrosis after discontinuation of chronic CCl₄ administration in rats. The exact mechanism for the increased apoptosis in the Se-supplemented mice remains uncertain. Pro-apoptotic BAX and Bcl Xs/l proteins were only increased after CCl₄ treatment in the Se-supplemented mice, while the anti-apoptotic protein Bcl-2 was not changed significantly. Cytosolic cytochrome c was not changed significantly by 4 weeks of CCl₄ treatment or Se supplementation. Most likely, the increase in cytosolic cytochrome c is an earlier event of CCl₄-induced hepatic injury. The Fas (CD95/APO-1) receptor which mediates apoptosis caused by a variety of insults [30], principally via the extrinsic death receptor pathway, was increased after CCl₄ treatment in the Se-supplemented mice. However, the activities of caspase 3 and caspase 8 were increased to a lesser extent after CCl₄ in the Se-supplemented mice.

Se is detoxified by successive methylation reactions catalyzed first by the enzyme thiol *s*-methyltransferase that converts hydrogen selenide to methylselenol and dimethylselenide, with subsequent conversion of dimethylselenide to trimethylselenonium catalyzed by thioether *s*-methyltransferase. Dimethylselenide is eliminated by the lung, while trimethylselenonium, which is water-soluble, is excreted in the urine [31]. Both selenium (selenite) and methylseleninic acid, an oxidation product of methylselenol, cause apoptosis in DU-145 cancer cells. While methylseleninic acid causes apoptosis via caspase 8 and the mitochondrial pathway with cytochrome c release, selenite causes apoptosis by a different pathway without those changes [32]. In this study, the TEMT was detected in the liver by Western blot and shown to be decreased by chronic CCl₄ administration regardless of Se supplementation. The lower TEMT concentration may result in increased retention of the methylated forms of selenium.

This study shows that Se supplementation decreased liver malondialdehyde in the control and CCl₄-treated mice. The hepatic damage caused by CCl₄ is due its metabolism to trichloromethyl (CCl₃·) and trichloromethylperoxyl (CCl₃OO·) reactive free radicals [33]. GSH defends against reactive free radicals by glutathione peroxidase enzymes which inactivate ROS via conversion of GSH to its oxidized form GSSG [6] or by donating an electron to the free radical R to form RH [34]. CCl₄ administration decreases liver GSH as we showed previously [5] and also decreases the Se-dependent glutathione peroxidase-enzyme, as shown in this study, allowing for the accumulation of free radical metabolites resulting in lipid peroxidation and liver damage. Oxidative stress is also well known to enhance collagen synthesis but can also enhance collagen degradation. Reactive oxygen species enhance stellate cell activation [1] and lipid peroxidation products, such as malondialdehyde, and stimulate α₁(I) collagen expression and collagen synthesis by stellate cells in culture [2,3]. ROS is generated by increased NAD(P)H oxidase which produces superoxide anion (O₂^{·-}) from oxygen (O₂), and the amount of hepatic fibrosis after chronic CCl₄ administration is less in NAD(P)H-deficient mice than in wild-type mice [4,5].

Oxidative stress, however, also increases active MMPs, which degrade collagen and other extracellular matrix proteins [35,36]. The most likely cause for the effect of Se supplementation in decreasing hepatic fibrosis caused by CCl₄ is a decrease in oxidant stress resulting in reduced collagen formation and increased collagen degradation. The postulated reduced collagen formation, which is associated with a lower number of stellate cells, may be due to a decrease in collagen translation and triple helix formation since it is not associated with a lower $\alpha_1(I)$ collagen mRNA.

In conclusion, this study shows that Se supplementation has a protective effect on hepatic fibrosis induced by CCl₄ in mice. The decreased hepatic fibrosis after chronic CCl₄ administration in mice supplemented with Se occurred in the setting of decreased inflammation but increased apoptosis. The principal mechanism for the decrease in fibrosis is a decrease in the number of collagen-producing stellate cells, which is accompanied by increased collagen degradation. Apoptosis of stellate cells is a mechanism for the lesser number of stellate cells after CCl₄ administration in Se-supplemented mice. Se deficiency in conjunction with enhanced oxidative stress, which is common in liver disease, most likely enhance liver injury and fibrosis caused by multiple agents such as alcohol and hepatitis C. Se supplementation in such cases could ameliorate liver injury and slow the progression of fibrosis.

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References

1. Nieto N, Friedman SL, Greenwel P, Cederbaum AI. CYP2E1-mediated oxidative stress induces collagen type I expression in rat hepatic stellate cells. *Hepatology* 1999;30:987–996. [PubMed: 10498651]
2. Chojkier M, Houghlum K, Solis-Herruzo J, Brenner DA. Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. A role for lipid peroxidation? *J Biol Chem* 1989;264:16957–16962. [PubMed: 2777815]
3. Maher JJ, Tzagarakis C, Gimenez A. Malondialdehyde stimulates collagen production by hepatic lipocytes only upon activation in primary culture. *Alcohol Alcohol* 1994;29:605–610. [PubMed: 7811345]
4. Novitskiy G, Potter JJ, Wang L, Mezey E. Influences of reactive oxygen species and nitric oxide on hepatic fibrogenesis. *Liver Int* 2006;26:1248–1257. [PubMed: 17105591]
5. Aram G, Potter JJ, Liu X, Torbenson MS, Mezey E. Deficiency of NAD(P) H oxidase enhances hepatocellular injury but attenuates fibrosis after chronic carbon tetrachloride administration. *Hepatology* 2009;49:911–919. [PubMed: 19072832]
6. Bedwal RS, Nair N, Sharma MP, Mathur RS. Selenium—its biological perspectives. *Med Hypotheses* 1993;41:150–159. [PubMed: 8231995]
7. Luoma PV, Sotaniemi EA, Korpela H, Kumpulainen J. Serum selenium, glutathione peroxidase activity and high density lipoprotein cholesterol—effect of selenium supplementation. *Res Commun Chem Pathol Pharmacol* 1984;46:469–472. [PubMed: 6515133]
8. Valimaki M, Alfthan G, Pikkarainen J, Ylikahri R, Salaspuro M. Blood and liver selenium concentrations in patients with liver diseases. *Clin Chim Acta* 1987;166:171–176. [PubMed: 3621598]

9. Thuluvath PJ, Triger DR. Selenium in chronic liver disease. *J Hepatol* 1992;14:176–182. [PubMed: 1500682]
10. Wenzel G, Kuklinski B, Ruhlmann C, Ehrhardt D. Alkoholtoxische hepatitis—‘eine frei Radikale’ assoziierte Erkrankung. Letalittssenkung durch adjuvante Antioxidantientherapie. *Inn Med* 1993;48:490–496.
11. Stewart S, Prince M, Bassendine M, Hudson M, James O, Jones D, Record C, Day CP. A randomized trial of antioxidant therapy alone or with corticosteroids in acute alcoholic hepatitis. *J Hepatol* 2007;47:277–283. [PubMed: 17532088]
12. Chen L, Pan DD, Zhou J, Jiang YZ. Protective effect of selenium enriched lactobacillus on CCl₄-induced liver injury in mice and its possible mechanisms. *World J Gastroenterol* 2005;11:5795–5800. [PubMed: 16270387]
13. Shen XH, Cheng WF, Li XH, Sun JQ, Li F, Ma L, Xie LM. Effects of dietary supplementation with vitamin E and selenium on rat hepatic stellate cell apoptosis. *World J Gastroenterol* 2005;11:4957–4961. [PubMed: 16124045]
14. Horwitz, W.; Latimer, GW., Jr., editors. Official methods of analysis of AOAC international. 18th edn. Gaithersburg; MD: 2005. p. 62–64. Chapter 4.
15. Potter JJ, Rennie-Tankersley L, Mezey E. Influence of leptin in the development of hepatic fibrosis produced in mice by *Schistosoma mansoni* infection and by chronic carbon tetrachloride administration. *J Hepatol* 2003;38:281–288. [PubMed: 12586293]
16. Bergmeyer HU, Scheibe P, Wahlefeld AW. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. *Clin Chem* 1978;24:58–73. [PubMed: 22409]
17. Uchiyama M, Mihara M. Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 1978;86:271–278. [PubMed: 655387]
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265–275. [PubMed: 14907713]
19. Hernandez-Munoz R, Diaz-Munoz M, Suarez-Cuenca JA, Trejo-Solis C, Lopez V, Sanchez-Sevilla L, Yanez L, Chagoya de Sanchez V. Adenosine reverses a preestablished CCl₄-induced micronodular cirrhosis through enhancing collagenolytic activity and stimulating hepatocyte cell proliferation in rats. *Hepatology* 2001;34:677–687. [PubMed: 11584363]
20. Zhou X, Hovell CJ, Pawley S, Hutchings MI, Arthur MJP, Iredale JP, Benyon RC. Expression of matrix metalloproteinase-2 and -14 persists during early resolution of experimental liver fibrosis and might contribute to fibrolysis. *Liver Int* 2004;24:492–501. [PubMed: 15482348]
21. Mezey E, Potter JJ, Slusser RJ, Abdi W. Changes in hepatic collagen metabolism in rats produced by chronic ethanol feeding. *Lab Invest* 1977;36:206–214. [PubMed: 557149]
22. Benyon RC, Arthur MJP. Extracellular matrix degradation and the role of hepatic stellate cells. *Semin Liver Dis* 2001;21:373–384. [PubMed: 11586466]
23. Zeng H, Briske-Anderson M, Idso JP, Hunt CD. The selenium metabolite methylselenol inhibits the migration and invasion potential of HT1080 tumor cells. *J Nutr* 2006;136:1528–1532. [PubMed: 16702316]
24. Kim SH, Johnson VJ, Shin TY, Sharma RP. Selenium attenuates lipopolysaccharide-induced oxidative stress responses through modulation of p38 MAPK and NF-κB signaling pathways. *Exp Biol Med* 2004;229:203–213.
25. Vunta H, Belda BJ, Arner RJ, Reddy CC, Vanden Heuvel JP, Prabhu KS. Selenium attenuates proinflammatory gene expression in macrophages. *Mol Nutr Food Res* 2008;52:1316–1323. [PubMed: 18481333]
26. Nolan JP, Leibowitz AI. Endotoxin and the liver. III. Modification of acute carbon tetrachloride injury by polymixin B—an antiendotoxin. *Gastroenterology* 1978;75:445–449. [PubMed: 210083]
27. Shen HM, Yang CF, Ong CN. Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG₂ cells. *Int J Cancer* 1999;81:820–828. [PubMed: 10328239]
28. Rikiishi H. Apoptotic cellular events for selenium compounds involved in cancer prevention. *J Bioenerg Biomembr* 2007;39:91–98. [PubMed: 17549643]
29. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Howell C, Arthur MJP. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and

- reduced hepatic expression of metalloproteinase inhibitors. *J Clin Invest* 1998;102:538–549. [PubMed: 9691091]
30. Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, Krammer PH, Runkel L. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. *J Exp Med* 1995;182:1223–1230. [PubMed: 7595193]
31. Wilson AC, Thompson HJ, Schedin PJ, Gibson NW, Ganther HE. Effect of methylated forms of selenium on cell viability and the induction of DNA strand breakage. *Biochem Pharmacol* 1992;43:1137–1141. [PubMed: 1554385]
32. Jiang C, Wang Z, Ganther H, Lu J. Caspases as key executors of methyl selenium-induced apoptosis (Anoikis) of DU-145 prostate cancer cells. *Cancer Res* 2001;61:3062–3070. [PubMed: 11306488]
33. Berk RF, Lane JM, Patel K. Relationship of oxygen and glutathione in protection against carbon tetrachloride-induced hepatic microsomal lipid peroxidation and covalent binding in the rat. Rationale for the use of hyperbaric oxygen to treat carbon tetrachloride ingestion. *J Clin Invest* 1984;74:1996–2001. [PubMed: 6511912]
34. Wardman, P. Conjugation and oxidation of glutathione via thiyl free radicals. In: Sies, H.; Ketterer, B., editors. *Glutathione conjugation. Mechanisms and biological significance*. Academic; London: 1988. p. 43-72.
35. Siwik DA, Pagano PJ, Colucci WS. Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. *Am J Physiol Cell Physiol* 2001;280:C53–C60. [PubMed: 11121376]
36. Leon H, Bautista-Lopez N, Sawicka J, Schultz R. Hydrogen peroxide causes cardiac dysfunction independent from its effect on matrix metalloproteinase-2 activation. *Can J Physiol Pharmacol* 2007;85:341–348. [PubMed: 17612643]

Fig. 1.

Effects of selenium supplementation (Se) on liver selenium, selenium-dependent glutathione peroxidase activity, thioether *s*-methyltransferase, and on liver malondialdehyde after 4 weeks of chronic carbon tetrachloride (CCl₄) administration. * $p < 0.05$ vs. control. ** $p < 0.01$ vs. control, + $p < 0.01$ vs. Se alone, ++ $p < 0.01$ vs. CCl₄

Fig. 2.

Effect of Selenium supplementation on necroinflammatory changes and on serum alanine aminotransferase after 4 weeks of chronic CCl₄. Liver cell necrosis (N) was evaluated on a scale of 1–4 as follows: 1 occasional, 2 mild scattered, 3 confluent zonal, and 4 extensive. Grades for hepatic inflammation (I) were: 1 scattered, 2 mild, 3 moderate, and 4 marked. The histological changes were assessed in $\times 20$ magnification fields of slides stained with hematoxylin–eosin. The values are expressed as means $\pm$ SE of eight determinations per group. * $p < 0.01$ vs. CCl₄ for grade of inflammation. * $p < 0.01$ vs. control or Se alone, respectively, for ALT

Fig. 3. Histology with Sirius red collagen staining of liver sections from mice treated for 4 weeks with **a** CCl₄ alone and **b** CCl₄ plus Se supplementation (original magnification $\times 100$). **c** Relative densitometry of fibrosis in the CCl₄ alone and CCl₄ + Se mice after chronic CCl₄ administration. The degree of fibrosis was measured by Sirius red staining and densitometry of the various groups. The density of fibrosis was determined as intensity of Sirius red staining divided by the area of the captured field. A total of 24 fields were captured from livers of each group of mice. The values are expressed as means $\pm$ SE. * $p < 0.01$ vs. respective control. + $p < 0.01$ vs. CCl₄. **d, e** Immunofluorescent detection of activated stellate cells after

CCl₄ treatment alone (**d**) or CCl₄ + Se (**e**). The liver slices were incubated with Cy3 monoclonal α -smooth muscle actin antibody (original magnification $\times 100$)

Fig. 4. Liver $\alpha_1(I)$ collagen mRNA in the CCl₄ alone and CCl₄ + Se mice after chronic CCl₄ administration. The mRNAs were determined by real-time quantitative PCR. The relative amounts of mRNA were normalized against β -actin in the same samples. The values are expressed as means $\pm$ SE of eight determinations per group. * p <0.01 vs. respective control

Fig. 5. Apoptosis determined by TUNEL staining of liver sections from mice after chronic CCl₄ administration. **a** CCl₄. **b** CCl₄ + Se. Original magnification $\times 100$

Fig. 6. Detection of stellate cell apoptosis in 5 μ m sections of cryopreserved liver tissue sections of mice after chronic CCl₄ administration and Se supplementation. Two different sections are shown as **a** and **b**. Apoptosis was determined by TUNEL staining (*green*) in stellate cells (*arrows*) identified with Cy3-conjugated monoclonal α -smooth muscle actin antibody (*red*). DAPI was used for nuclear staining (*blue*). The immunostained slices were evaluated and photographed with laser confocal microscopy (original magnification $\times 1,200$)

Fig. 7.

Effects of chronic CCl₄ with and without Se supplementation on Fas and on activities of caspases 3 and 8. FAS was determined by Western blot and quantitated by densitometry. All values are expressed as means $\pm$ SE of eight measurements per group. * p <0.05 vs. CCl₄ for FAS. * p <0.05 vs. control and ⁺ p <0.05 vs. CCl₄ for caspase 8 activity

Fig. 8. Effects of chronic CCl₄ administration with and without Se supplementation on Bax, Bcl Xs/l, Bcl-2 and on cytosolic cytochrome c. The determinations were done by Western blot. The values are expressed as means±SE of relative densitometry of eight measurements per group. * $p < 0.05$ vs. CCl₄

Fig. 9. Effects of chronic CCl₄ administration on matrix metalloproteinase 2 (MMP-2), 9 (MMP-9), tissue inhibitor of metalloproteinase 1 (TIMP-1), and TIMP-2 determined by Western blot. * $p < 0.05$ vs. CCl₄