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Hyperhomocysteinemia induces liver injury in rat: Protective effect of folic acid supplementation

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Abstract

Hyperhomocysteinemia, a condition of elevated blood homocysteine level, is an independent risk factor for cardiovascular diseases. Hyperhomocysteinemia is also found in patients with liver diseases. However, the direct effect of homocysteine on liver injury is not well known. Folic acid supplementation is a promising approach for improving endothelial function in patients with hyperhomocysteinemia. The aim of this study was to investigate the direct effect of hyperhomocysteinemia on liver injury and whether folic acid could offer any protective effect to the liver. Hyperhomocysteinemia was induced in rats fed a high-methionine diet for 4 weeks. There was a significant increase in the serum aspartate aminotransferase and alanine aminotransferase activities reflecting liver injury in hyperhomocysteinemic rats. Hepatic NAD(P)H oxidase was activated during hyperhomocysteinemia leading to increased superoxide anion production and peroxynitrite formation in the liver. As a consequence, the level of lipid peroxides was significantly elevated in livers of hyperhomocysteinemic rats. Folic acid supplementation effectively inhibited NAD(P)H oxidase-mediated superoxide anion production leading to reduced lipid peroxidation in the liver. Folic acid supplementation also alleviated hyperhomocysteinemia-induced liver injury. These results suggest that hyperhomocysteinemia can cause liver injury and supplementation of folic acid offers a hepatoprotective effect.

Keywords: Homocysteine; Folic acid; Oxidative stress; Liver function

1. Introduction

Hyperhomocysteinemia is regarded as an independent risk factor for cardiovascular diseases [1–3]. It is defined as plasma or serum homocysteine (Hcy) levels higher than 15 μM [3]. Hcy is an intermediate amino acid formed during the metabolism of methionine. Abnormal elevations of plasma Hcy levels up to 100–250 μM have been reported in patients with severe hyperhomocysteinemia due to genetic defects of enzymes that are involved in Hcy metabolic pathways [3]. Recent evidence suggests that hyperhomocysteinemia is also associated with diseases that involve other organs [4,5]. In an early study, McCully observed extensive arteriosclerosis in a pediatric patient with severe hyperhomocysteinemia and proposed a pathogenic link between elevated blood Hcy levels and atherogenesis [6]. The necropsy also revealed moderately fatty liver [6]. Subsequent investigations demonstrated an association between hyperhomocysteinemia and liver disease [4,7]. Plasma Hcy levels are often elevated in patients with liver cirrhosis or chronic alcohol-induced liver injury due to impaired Hcy metabolism [4,7]. Our recent study demonstrated an early sign of hepatic steatosis (fatty liver) in hyperhomocysteinemic rats [8]. Hyperhomocysteinemia caused an activation of several transcription factors in the liver leading to increased HMG-CoA reductase and cholesterol biosynthesis [8]. As a consequence, hepatic lipid accumulation and hypercholesterolemia occurred [8].

Oxidative stress due to excessive generation of reactive oxygen species (ROS) has been suggested as one of the

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important mechanisms for Hcy-induced cardiovascular injury [9,10]. In our previous study, we observed that hyperhomocysteinemia could induce excessive superoxide anion generation and expression of inflammatory markers as well as impairment of endothelium-dependent vessel relaxation [9,11]. Superoxide anion is a reactive oxygen free radical that can rapidly interact with nitric oxide (NO) to form highly reactive peroxynitrite, a potent oxidant that can cause tissue damage [12–14]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the primary enzyme for superoxide anion generation [15]. The activation of NAD(P)H oxidase leads to a massive production of reactive oxygen species (ROS) including superoxide anion as an initial product. Increased superoxide anion production is thought to be one of the important mechanisms contributing to liver injury [16,17]. Inhibition of NAD(P)H oxidase expression protected the liver against alcohol-induced injury in rodent models [16,17].

Hcy can be metabolized via two major pathways, namely, remethylation pathway and transsulfuration pathway. In the remethylation pathway, Hcy can be converted to methionine catalyzed by methionine synthase with folate as a co-substrate or catalyzed by betaine-Hcy methyltransferase using betaine as a co-substrate. In the transsulfuration pathway, Hcy is irreversibly converted to cystathionine by cystathionine β-synthase (CBS). Factors that perturb the steps in Hcy metabolic pathways can cause an increase in cellular Hcy levels and lead to its elevation in the blood [3,18]. Folic acid is a synthetic form of folate that is a water soluble B vitamin. The active metabolite of folic acid is 5-methyltetrahydrofolate that facilitates the remethylation of Hcy to methionine. Oral folic acid supplementation has been shown to reduce plasma Hcy levels as well as improve the endothelial function in individuals with mild hyperhomocysteinemia [19]. We previously observed that hyperhomocysteinemia could be induced in rats fed a high methionine diet for 4 weeks [8,9,11]. In this animal model, folic acid supplementation abolished Hcy-induced expression of chemokines and adhesion molecules in the aortic endothelium [11].

At present, the direct effect of hyperhomocysteinemia on liver injury is not well known. Although folic acid supplementation is viewed as a promising approach for the prevention and treatment of cardiovascular disease associated with hyperhomocysteinemia or with other risk factors [19–21], its role in liver injury remains to be examined. In this study, we investigated the effect of hyperhomocysteinemia and folic acid supplementation on hepatic oxidative stress and liver injury in hyperhomocysteinemic rats.

2. Materials and methods

2.1. Animal model

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) aged 8 weeks were divided into five groups and maintained for 4 weeks on the following diets: (1) control diet (regular diet) consisting of Lab Diet Rodent Diet 5001 (PMI Nutrition International, St. Louis, MO, USA) which contained 0.43% (wt/wt, 4.3 g/kg) methionine, 0.00059% (wt/wt, 5.9 mg/kg) folic acid and 0.0006% (wt/wt, 6 mg/kg) vitamin B_6; (2) high-methionine diet consisting of regular diet plus 1.7% (wt/wt) methionine; and (3) high-methionine plus folic acid diet, consisting of regular diet plus 1.7% (wt/wt) methionine and 0.025% (wt/wt, 250 mg/kg) folic acid; (4) high-methionine plus folic acid and vitamin B_6 diet, consisting of regular diet plus 1.7% (wt/wt) methionine, 0.025% (wt/wt) folic acid and 0.006% (wt/wt, 60 mg/kg) vitamin B_6; (5) high-methionine plus vitamin B_6 diet, consisting of regular diet plus 1.7% (wt/wt) methionine and 0.006% (wt/wt) vitamin B_6 [8,9,11,22]. Results from our previous studies demonstrated that hyperhomocysteinemia could be induced in rats after 4 weeks of high-methionine diet [8,9,11]. The Hcy and folate concentrations in the serum were measured with the IMx assays (Abbott Diagnostics, Abbott Park, IL, USA) and DELFIA® Folate kit (PerkinElmer, Boston, MA, USA), respectively [8,9,11]. The liver injury was assessed by measuring the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum samples with enzymatic kits (Wako Chemicals, Richmond, VA, USA). All procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals published by Canadian Council on Animal Care and approved by University of Manitoba Protocol Management and Review Committee.

2.2. Immunohistochemical staining

Immunohistochemical staining for nitrotyrosine protein adducts was performed. In brief, the liver was excised and a portion of it was immersion-fixed in 10% neutral-buffered formalin overnight followed by embedding in paraffin. Sequential 5 μm paraffin-embedded cross sections were prepared [8,23]. Sections were stained with nitrotyrosine protein adducts [23]. In brief, sections were incubated with 2% BSA blocking agent following permeabilization. Mouse anti- nitrotyrosine antibodies (1:100) (Zymed Laboratories, South San Francisco, CA, USA) were used as primary antibody. Sections were treated with 0.3% hydrogen peroxide (H_2O_2) for 20 min at room temperature to inhibit the endogenous peroxidase. The sections were then incubated with biotin-conjugated anti-mouse immunoglobulins (1:200, DakoCytomation, Carpinteria, CA, USA) as secondary antibodies followed by peroxidase conjugated-streptavidin (Zymed). Sections were then treated with 3,3-diaminobenzidine (DAB)-H_2O_2 colorimetric substrate solution. The attached peroxidase catalyzed the H_2O_2-mediated oxidation of DAB to yield an insoluble brown precipitate. The area displayed brownish color indicating the nitrotyrosine protein adducts [23]. The images (5 per liver) were captured and examined using an Axioskop2 MOT microscope (Carl Zeiss Microimaging, Thornwood, NY, USA), an Axiocam camera, and Photoshop 6.0 (Adobe, San Jose, CA, USA).

2.3. Determination of NAD(P)H oxidase activity and antioxidant enzyme activities

The NAD(P)H oxidase activity was measured by lucigenin chemiluminescence's assay [24]. A portion of the liver was homogenized in a 50 mM phosphate buffer (pH 7.0, 1:10, w/v) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 3000×*g* for 10 min, an aliquot of the supernatant (100 μg proteins) were incubated with lucigenin (5 μM) in a phosphate buffer (50 mM, pH 7.0) for 2 min followed by adding the substrate, 100 μM NADPH [24]. Chemiluminescent signal (photon emission) was measured every 15 s for 3 min using a luminometer (Lumet LB9507, Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany). In principle, reaction of lucigenin with superoxide anion leads to the formation of lucigenin dioxetane that decomposes to produce two molecules of N-methylacridone [25]. One of these two N-methylacridone molecules is in an electronically excited state and emits a photon. The photon emission that reflects the amount of superoxide anion in the sample can be detected using a luminometer [25]. A standard curve was prepared with xanthine (100 μM) and known serial concentrations of xanthine oxidase (Sigma-Aldrich, St. Louis, MO, USA). The NAD(P)H oxidase activity was calculated based on the amount of superoxide anion produced in the reaction mixture. To verify that the photon signal detected in livers of hyperhomocysteinemic rats was mainly generated from NAD(P)H oxidase, the same experiments were performed using sodium succinate, arginine or xanthine as a substrate in the lucigenin chemiluminescence assay. In assays using those substrates, a negligible chemiluminescent signal was detected. In addition, hepatic superoxide

dismutase (SOD), catalase and glutathione peroxidase activities (GPx) were determined as previously described [26,27].

2.4. Determination of superoxide anion

The level of superoxide anion in the liver was determined as previously described with minor modification [9]. A portion of the liver was homogenized in a buffer (1:4, w/v) containing 20 mM HEPES, 1 mM EDTA and 0.1 mM PMSF. After centrifugation at 3000×*g* for 10 min, an aliquot of supernatant was incubated in a reaction mixture containing 10 μM dihydroethidium for 30 min at 37 °C. The superoxide anion in the liver homogenate caused the oxidation of dihydroethidium leading to the formation of ethidium which was detected at an excitation of 475 nm and an emission of 610 nm using a fluorometer (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA, USA). The fluorescent signal produced by ethidium was proportional to the level of superoxide anion present in the liver.

2.5. Determination of nitric oxide synthase (NOS) activity and NO metabolites

Total NOS activity in the liver was measured using L-citrulline assay [28]. In brief, a portion of the liver was homogenized in a lysis buffer (1:4 w/v) containing 50 mM Tris, 0.1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.1 mM EDTA, 2 μM leupeptin, 1 mM PMSF, 1% (v/v) Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.1% deoxycholate, pH 7.5 followed by centrifugation at 3000×*g* for 10 min [28]. An aliquot of the supernatant was incubated in a reaction mixture containing 50 mM Tris, 1 mM NADPH, 3 μM tetrahydrobiopterin, 100 mM calmodulin, 2.5 mM $CaCl_2$, 50 mM L-valine, 10 μM L-arginine and 0.2 μCi L-[^{3}H]-arginine as a substrate at 37 °C for 1 h. The reaction was stopped by adding a cold stopping buffer including 20 mM HEPES, 2 mM EDTA and 2 mM EGTA, pH 5.5. The radiolabeled reaction product, L-citrulline was purified by anion exchange chromatography with Dowex AG 50WX-8 resin column (Bio-Rad, Hercules, CA, USA). The radioactivity associated with L-citrulline was measured with a scintillation counter and the NOS activity was calculated [28]. The measurement of nitrite and nitrate was used to assess the NO levels in liver tissue [29]. In brief, a portion of the liver was homogenized in a buffer containing 20 mM Tris, 2 mM EDTA, pH 7.4. After deproteinization, the amount of nitrite and nitrate was determined with Griess reaction method based on the azo coupling reaction [29]. In brief, the supernatant was incubated with nitrate reductase to reduce nitrate to nitrite. The 12.5 mM sulfanilamide in 6 M HCl and 12.5 mM N-(1-naphthyl) ethylenediamine were then added to the reaction to complete the azo coupling reaction. The diazoamino benzene formed in the reaction mixture was measured by spectrophotometer at absorbance of 520 nm. The $NaNO_2$ at different concentrations was used as standards.

2.6. Determination of lipid peroxidation

The degree of lipid peroxidation in the liver tissue was determined by measuring thiobarbituric acid reactive substances (TBARS) [30,31]. Briefly, a portion of the liver was homogenized in 1.14% KCl solution containing 50 mM desferroxamine in 1:10 volumes followed by centrifugation at 3000×*g* for 10 min at 4 °C. An aliquot of supernatant (0.1 mL) was added to the reaction mixture containing 0.1 mL 8% SDS, 0.375 mL 20% acetic acid, 0.375 mL 0.8% thiobarbituric acid and 0.1 mL water. After incubation at 95 °C for 1 h, the amount of malondialdehyde (MDA) formed in the reaction mixture was measured by spectrophotometer at absorbance of 532 nm. The MDA was used as the standard and the results were expressed as nmol MDA produced per mg protein. The amount of MDA correlated to the level of lipid peroxides in the liver.

2.7. Statistical analysis

Results were analyzed by a two-tailed Student's *t*-test or using one-way analysis of variance (ANOVA) followed by Pearson's correlation test. Data were presented as the means±S.E.M. *P* values less than 0.05 were considered significant.

3. Results

3.1. Induction of hyperhomocysteinemia and detection of liver injury

Hyperhomocysteinemia was induced in rats fed a high-methionine diet for 4 weeks. A significant increase in serum Hcy concentrations was detected in this group of rats (25.48 μM versus 3.52 μM in control) (Table 1). Supplementation of folic acid to rats fed a high-methionine diet lowered the serum Hcy levels from 25.48 μM to 18.66 μM (Table 1). The serum folic acid concentration in high-methionine plus folic acid fed group was 2.9-fold higher than that in the control or in high-methionine fed group (Table 1). To determine whether hyperhomocysteinemia resulted in liver injury, the serum aminotransferase activities were measured. The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in hyperhomocysteinemic rats were significantly elevated, indicating liver injury (Table 1). Supplementation of folic acid to rats fed a high-methionine diet effectively alleviated liver injury as demonstrated by a reduction of both AST and ALT activities (Table 1). These results suggested that 4-week exposure to hyperhomocysteinemia was able to cause liver injury and folic acid supplementation had a protective effect against Hcy-induced liver injury.

3.2. Increased NAD(P)H oxidase dependent superoxide production in the liver

The NAD(P)H oxidase activity was significantly increased in livers of hyperhomocysteinemic rats (Fig. 1A). In accordance, there was a significant elevation of superoxide anion levels in

Table 1
Serum concentrations of Hcy and folate, and aminotransferase activities

Diet	Hcy (μM)	Folate (ng/mL)	AST (IU/L)	ALT (IU/L)
Control	3.52±0.34	89.43±7.13	26.62±3.82	36.28±6.06
High-methionine	25.48±3.01*	89.70±7.58	42.89±7.60*	60.62±9.54*
High-methionine plus Folic acid	18.66±1.39*#	255.94±24.94*#	34.07±4.47	45.39±4.10
High-methionine plus Apocynin	26.28±5.84*	86.21±15.49	28.56±2.08	42.82±8.75

Rats were fed with following diets for 4 weeks: a regular diet (Control), a high methionine diet (1.7%) or a high methionine plus folic acid diet (n=12 for each group). One group of high-methionine fed rats was given IP injection of apocynin (4 mg/kg, daily) for 3 days prior to euthanasia (n=8). Values were expressed mean±S.E.M. *P<0.05 compared with control values. #P<0.05 compared with values of high-methionine treated group. Liver injury was examined by measuring the enzyme activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

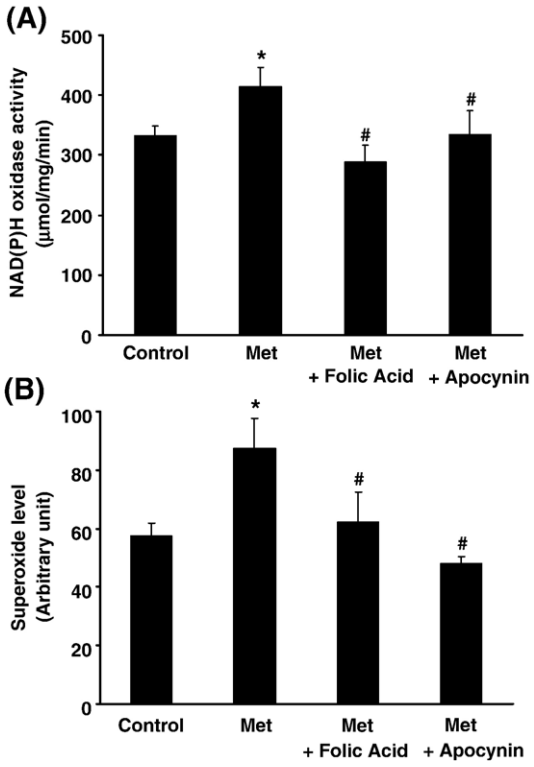

Fig. 1. NAD(P)H oxidase activity and superoxide anion content in rat livers. Rats were fed with following diets for 4 weeks: a regular diet (Control), a high methionine diet (1.7%, Met), or a high methionine plus folic acid diet (Met+Folic acid) ($n=12$ for each group). One group of high-methionine fed rats was given IP injection of apocynin (4 mg/kg, daily) for 3 days prior to euthanasia (Met+Apocynin) ($n=8$). Livers were isolated and (A) NAD(P)H oxidase activity (expressed as the amount of superoxide anion produced per mg of protein per min) and (B) the superoxide anion level in the liver tissue was determined. Results were expressed as mean±S.E.M. *$P<0.05$ compared with control values. $^{\#}P<0.05$ compared with values of high-methionine treated group.

livers of hyperhomocysteinemic rats (Fig. 1B). Folic acid supplementation reduced the NAD(P)H oxidase activity and the superoxide anion content to the basal levels (Fig. 1). Treatment of hyperhomocysteinemic rats with apocynin, an inhibitor for NADPH oxidase [16], completely blocked hyperhomocysteinemia-induced NAD(P)H oxidase activation and elevation of superoxide anion levels in the liver (Fig. 1). Folic acid supplementation also reversed liver injury as demonstrated by a reduction of both AST and ALT activities (Table 1). Similar results were observed in hyperhomocysteinemic rats treated with apocynin (Table 1). To test whether increased superoxide anion production was derived from the mitochondrial respiration system, experiments were performed by adding rotenone (an inhibitor of mitochondrial respiration) to the lucigenin chemiluminescence assay mixture [25]. Such treatment did not affect hyperhomocysteinemia-induced superoxide anion generation in the liver tissue (data not shown). These results suggested that hyperhomocysteinemia-induced liver injury might be mediated via oxidative stress due to increased NAD(P)H oxidase dependent superoxide anion production. Folic acid supplementation exerted a protective effect against liver injury.

3.3. Increased nitrotyrosine formation in the liver during hyperhomocysteinemia

Superoxide anion can react with nitric oxide (NO) to form peroxynitrite. To determine whether there was an increase in peroxynitrite formation in the liver during hyperhomocysteinemia, immunohistochemical analysis was performed to detect nitrotyrosine, a biomarker for peroxynitrite [32]. Little nitrotyrosine protein adducts were detected in the liver samples isolated from control rats (Fig. 2A). In contrast to the control, a significant increase in the intensity of nitrotyrosine protein adduct staining was found in the livers of hyperhomocysteinemic rats (Fig. 2B), indicating an increased peroxynitrite formation. Rats treated with folic acid supplementation displayed much less staining of nitrotyrosine protein adducts in the liver samples (Fig. 2C). Treatment of hyperhomocysteinemic rats with apocynin completely blocked hyperhomocysteinemia-induced nitrotyrosine formation in the liver (Fig. 2D). Next, to determine whether hyperhomocysteinemia also stimulated NO production in the liver, the level of NO metabolites (nitrite and nitrate) and the NOS activity were measured. There was no change in the NO metabolite levels (Fig. 3A) or the NOS activity (Fig. 3B) in the livers of hyperhomocysteinemic rats. These results indicated that increased peroxynitrite formation in the liver during hyperhomocysteinemia was mainly due to excessive superoxide anion generation. Folic acid supplementation could antagonize Hcy-induced peroxynitrite formation (Fig. 2C).

3.4. Increased hepatic lipid peroxidation during hyperhomocysteinemia

Peroxynitrite is a potent oxidant and can cause lipid peroxidation, which is detrimental to tissues [33]. The degree of lipid peroxidation in the liver was examined by measuring the level of malondialdehyde (MDA), an indicator of lipid peroxidation. The MDA level was significantly elevated in the livers of hyperhomocysteinemic rats, reflecting an increased lipid peroxidation in the liver (Fig. 4). Folic acid supplementation reduced the liver MDA level to the value similar to that observed in the control rats (Fig. 4). Apocynin treatment also reduced the MDA level in hyperhomocysteinemic rats to the basal level (Fig. 4). These results suggested that NAD(P)H oxidase-mediated superoxide anion generation was responsible for oxidative stress and subsequently increased lipid peroxidation during hyperhomocysteinemia.

3.5. Decrease in hepatic antioxidant enzyme activities during hyperhomocysteinemia

Oxidative stress might also be a result of a decrease in antioxidant enzyme activities. Therefore, several antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were examined. As shown in Fig. 5, the activity of hepatic SOD and catalase was significantly decreased in hyperhomocysteinemic rats while the glutathione peroxidase (GPx) activity remained unchanged during hyperhomocysteinemia.

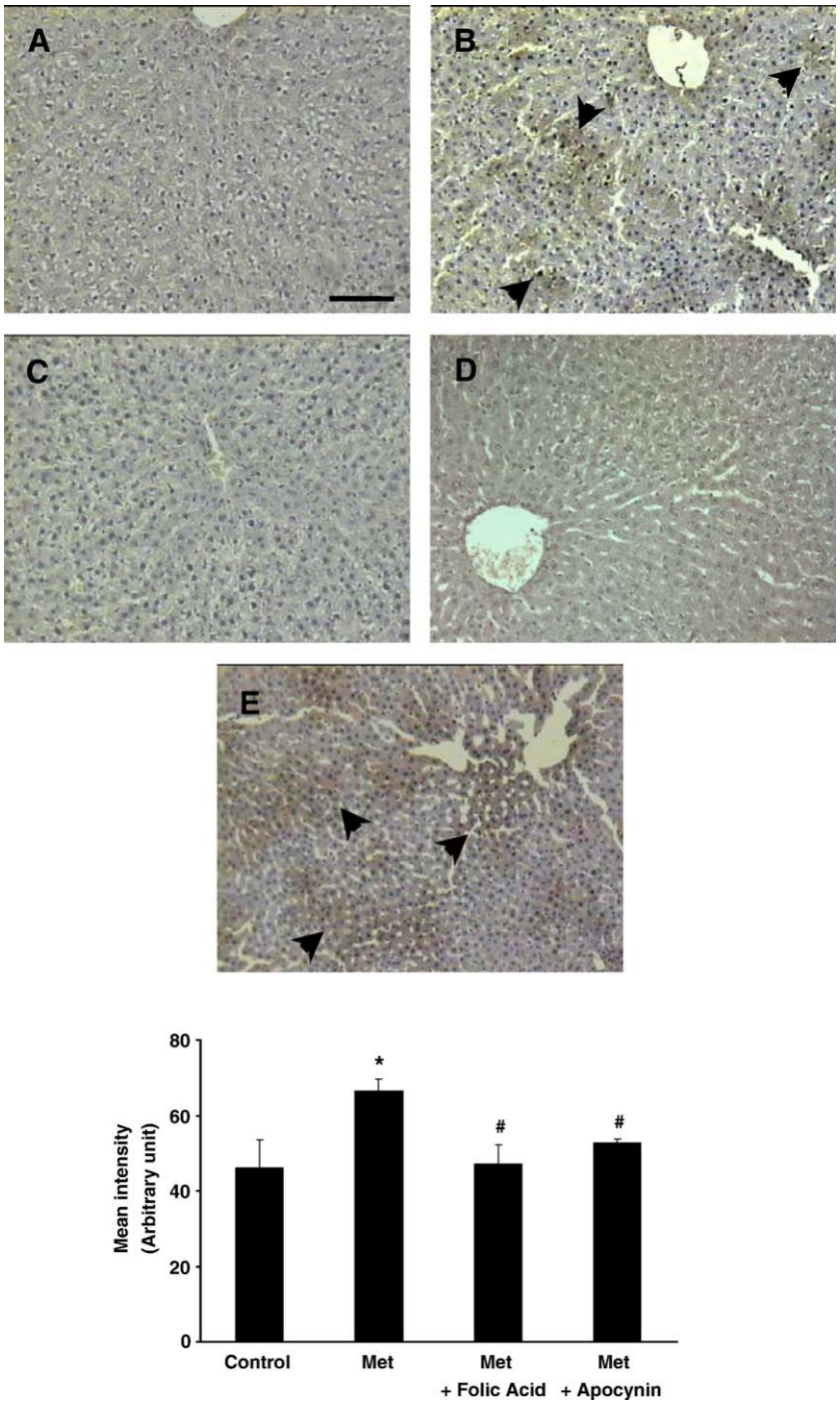

Fig. 2. Immunohistochemical staining of nitrotyrosine in rat livers. Livers were fixed in 10% neutral-buffered formalin overnight and then embedded in paraffin. Immunohistochemical staining for nitrotyrosine protein adducts was performed with anti-nitrotyrosine antibodies. After counterstaining with Mayer's hematoxylin, nitrotyrosine was identified under light microscope with a magnification of 200×. Representative photos were obtained from rats fed (A) a regular diet (Control), (B) high-methionine diet (1.7%, Met), (C) high-methionine plus folic acid diet (Met+Folic acid) ($n=12$ for each group), and (D) high-methionine plus IP injection of apocynin (4 mg/kg, daily) for 3 days prior to euthanasia (Met+Apocynin) ($n=8$). (E) The positive control was prepared from rats with IP injection of 300 mg/kg acetaminophen 6 h prior to euthanasia. Arrows point to the areas positively stained with nitrotyrosine. The intensity of stained area for nitrotyrosine was quantified using Photoshop 6.0 (bottom panel). The results were expressed as mean±S.E.M. $^{*}P<0.05$ compared with control values. $^{\#}P<0.05$ compared with values of high-methionine treated group (Scale bar=20 μm).

3.6. Effects of vitamin B_6 supplementation alone or in combination with folic acid

The effect of vitamin B_6 supplementation was examined. Vitamin B_6 supplementation to rats fed a high-methionine diet lowered the serum Hcy level from 25.48 μM to 17.17 μM (Fig. 6A). Combination of folic acid with vitamin B_6 did not produce further reduction in the serum Hcy level. Inhibition of hepatic NAD(P)H oxidase activity and MDA production were observed in rats supplemented with folic

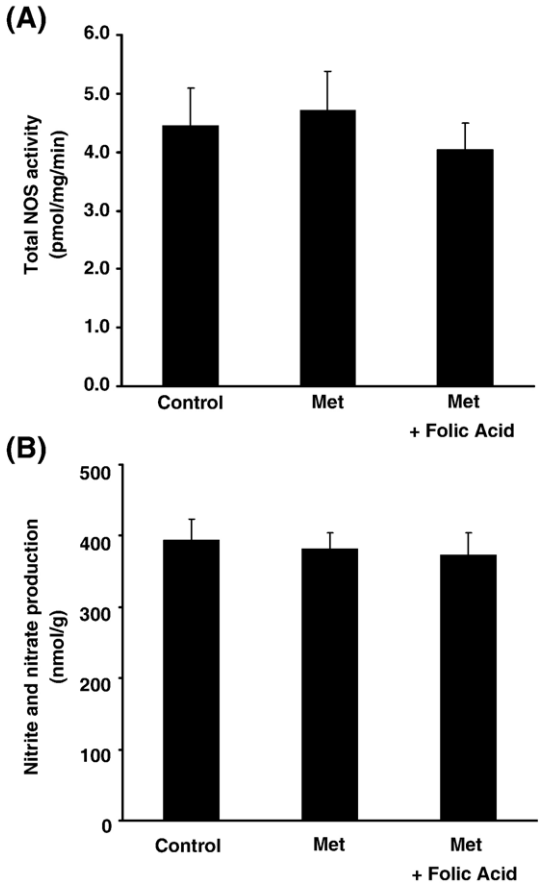

Fig. 3. Hepatic NOS activity and NO production. Rats were fed with following diets for 4 weeks: a regular diet (Control), a high methionine diet (1.7%, Met), or a high methionine plus folic acid diet (Met+Folic acid). (A) The total hepatic NOS activity in the liver was determined and expressed as the amount of L-citrulline produced per mg of protein per minute. (B) The levels of NO metabolites (nitrite and nitrate) were measured and expressed as the amount of nitrite and nitrate per g of wet weight of liver tissue. Results were expressed as mean±S.E.M. (n=12).

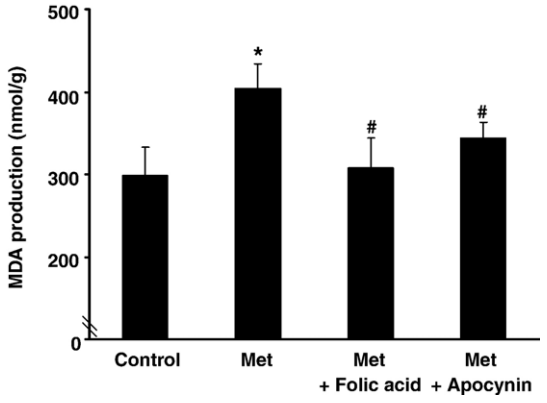

Fig. 4. Determination of lipid peroxidation in rat livers. Rats were fed with following diets for 4 weeks: a regular diet (Control), a high methionine diet (1.7%, Met), a high methionine plus folic acid diet (Met+Folic acid) (n=12 for each group) or high-methionine plus 3 days treatment of apocynin (Met+Apocynin) (n=8). Lipid peroxides (TBARS) in the livers was determined by measuring the amount of MDA in the liver tissue. The level of MDA was expressed as nmol per g of protein. Results were expressed as mean±S.E.M. *P<0.05 compared with control values. $^{\#}P$<0.05 compared with values of high-methionine treated group.

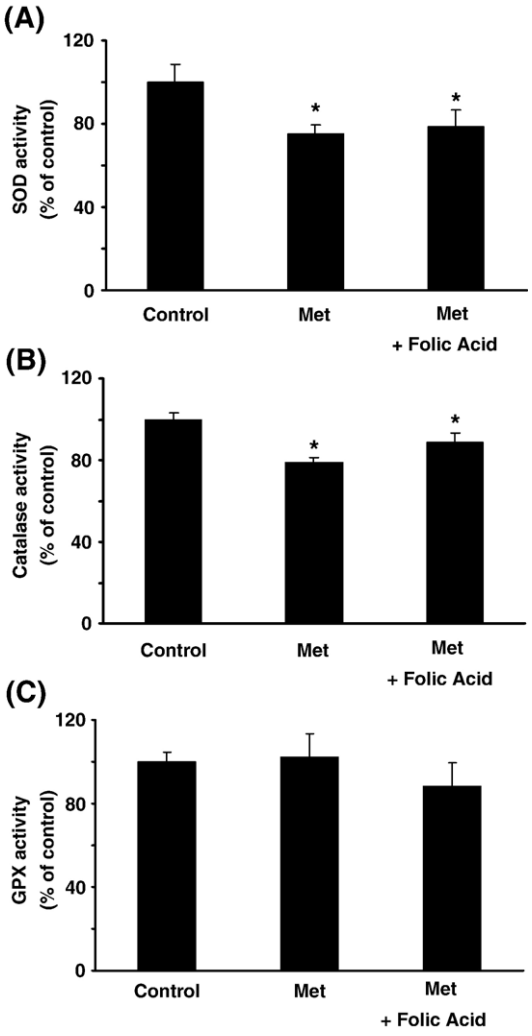

Fig. 5. Hepatic antioxidant enzymes activities. Rats were fed with following diets for 4 weeks: a regular diet (Control), a high methionine diet (1.7%, Met), a high methionine plus folic acid diet (Met+Folic acid) (n=12 for each group) or high-methionine plus 3 days treatment of apocynin (Met+Apocynin) (n=8). (A) Superoxide dismutase (SOD), (B) catalase and (C) glutathione peroxidase (GPx) activities were determined in the liver tissue and expressed as percentage of control. Results were expressed as mean±S.E.M. *P<0.05 compared with control values.

acid, vitamin B_6 or folic acid combined with vitamin B_6 (Fig. 6B, C). Liver injury was further examined by measuring serum AST and ALT enzyme activities. Supplementation of folic acid or folic acid combined with vitamin B_6 was able to reduce AST and ALT activities (data not shown).

3.7. Correlation between serum Hcy concentrations and oxidative stress

To examine the correlation between serum Hcy concentrations and oxidative stress, hyperhomocysteinemia was induced in rats fed 0.8% methionine or 1.7% methionine. There was a significant elevation of serum Hcy level in rats fed 0.8% methionine (7.47 μM vs. 3.52 μM in the control group) (Fig. 7A). In rats fed 1.7% methionine, the serum

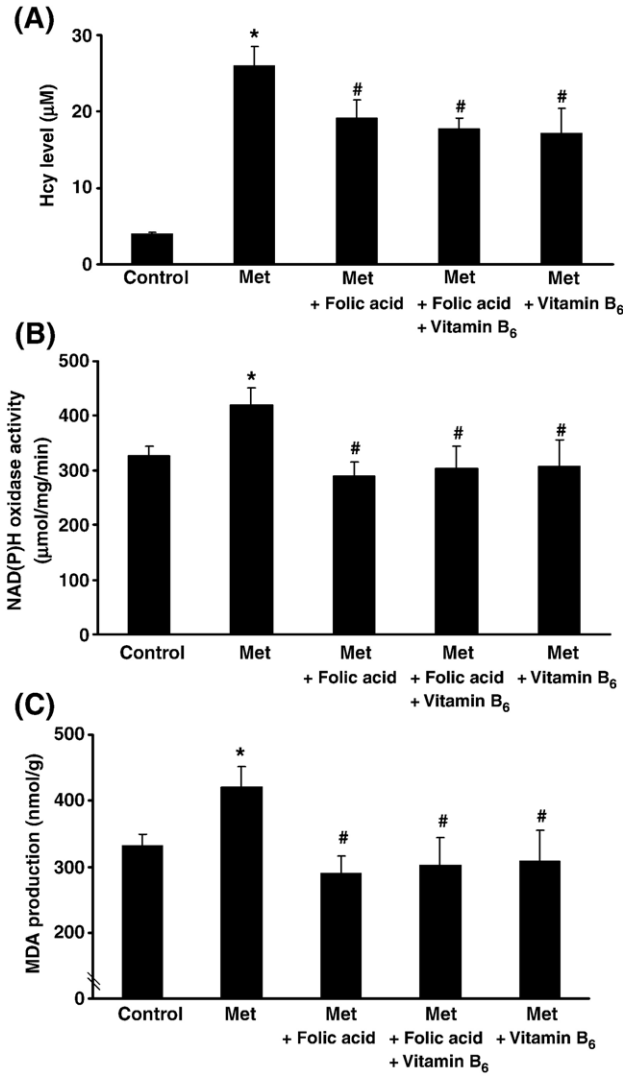

Fig. 6. The protective effects of folic acid and vitamin B_6. Rats were fed with following diets for 4 weeks: a regular diet (Control), a high methionine diet (1.7%, Met), a high methionine plus folic acid diet (Met+Folic acid), a high-methionine plus folic acid and vitamin B_6 (Met+Folic acid+Vitamin B_6) or a high-methionine plus vitamin B_6 (Met+Vitamin B_6). (A) Serum Hcy levels were determined. (B) The NAD(P)H oxidase activity were measured and expressed as the amount of superoxide anion produced per mg of protein per min. (C) Lipid peroxides (TBARS) in the livers were determined by measuring the amount of MDA in the liver tissue. Results were expressed as mean±S.E.M. $^{*}P<0.05$ compared with control values. $^{\#}P<0.05$ compared with values of high-methionine treated group.

Hcy level was elevated to 25.48 μM. The degree of lipid peroxidation in livers of those rats was examined. As shown in Fig. 7B, there was a significant increase in lipid peroxidation in livers of rats fed 1.7% methionine. However, the degree of lipid peroxidation in livers of 0.8% methionine-fed rats was similar to that found in the control group (Fig. 7B). Further analysis revealed that there was a significant correlation between the serum Hcy concentration and the hepatic superoxide anion level in rats fed high-methionine diet (Fig. 8A). The correlation coefficient was further increased when data with serum Hcy concentrations higher than 19 μM were analyzed (Fig. 8B). However, when data with serum Hcy concentration less than 19 μM were subjected to the same analysis, the correlation coefficient value was 0.22 and no significance was observed (Fig. 8C).

4. Discussion

The novel findings of this study are that (1) in the absence of other risk factors, diet-induced hyperhomocysteinemia alone can cause liver injury due to oxidative stress; (2) folic acid supplementation can reduce superoxide anion generation and alleviate hyperhomocysteinemia-induced liver injury.

Oxidative stress due to NAD(P)H oxidase-mediated superoxide anion generation is thought to be one of the important mechanisms for Hcy-induced cardiovascular dysfunction [9]. NAD(P)H oxidase is abundantly expressed in phagocytic cells and plays a major role in immune defense against microorganism invasion. The NAD(P)H oxidase is also expressed in non-phagocytic cells. The ROS generated by non-phagocytic NAD(P)H oxidase are involved in cell signaling, proliferation, apoptosis and cellular senescence [34]. The phagocytic NAD(P)H oxidase consists of cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and rac) and membrane-bound subunits ($p22^{phox}$ and $gp91^{phox}$) [35,36]. In non-phagocytic cells, homologues of $gp91^{phox}$ subunit have been identified including Nox and Duox [35]. Nox and Duox mRNA have been found in rat hepatocytes [37]. Several studies suggest that Kupffer cell (liver residing macrophage)-derived ROS exacerbates liver

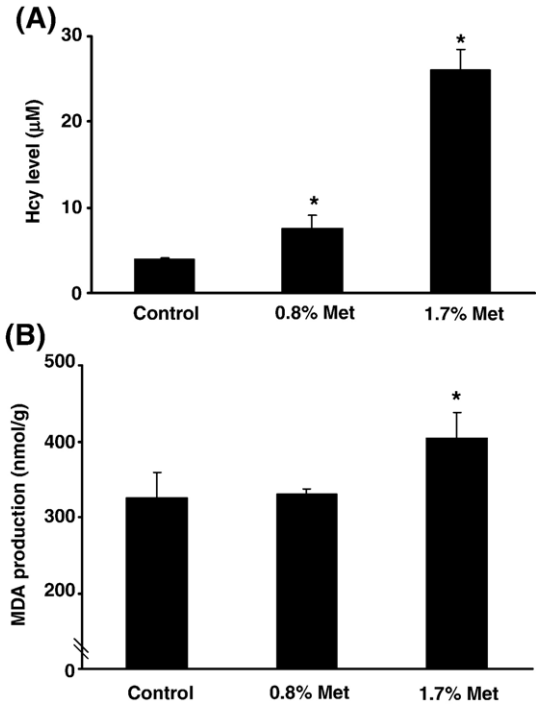

Fig. 7. Determination of hepatic lipid peroxidation. Rats were fed with following diets for 4 weeks: a regular diet (Control), a 0.8% methionine diet (0.8% Met), and a 1.7% methionine diet (1.7% Met) ($n=4$ for each group). (A) Serum Hcy levels were determined. (B) Lipid peroxides (TBARS) in the livers were determined by measuring the amount of MDA in the liver tissue. Results were expressed as mean±S.E.M. $^{*}P<0.05$ compared with control values.

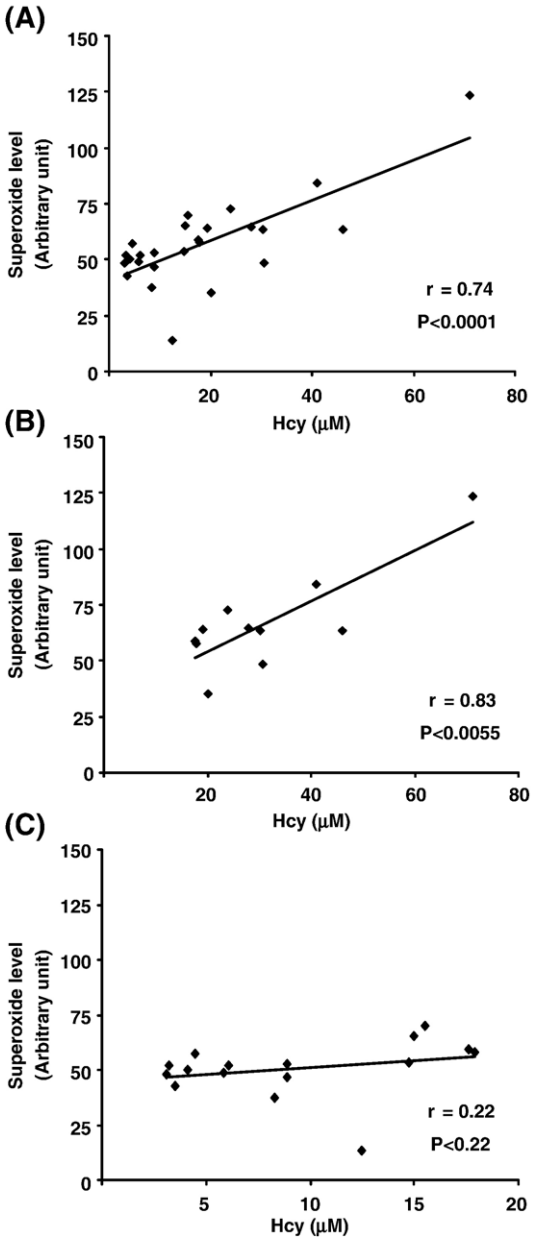

Fig. 8. Correlation of serum Hcy levels and hepatic superoxide anion content. Pearson's correlation between serum Hcy levels and hepatic superoxide anion content in (A) samples with the whole range of serum Hcy levels; (B) serum Hcy levels above 19 μM and (C) serum Hcy levels below 19 μM were analyzed. Both *r* and *P* values were calculated.

injury [38,39]. A recent study has found that hydrophobic bile acids can activate NAD(P)H oxidase in hepatocytes leading to oxidative stress mediated apoptosis [40]. Several lines of evidence from the present study suggested that hyperhomocysteinemia-induced liver injury was mediated via oxidative stress. First, there was a significant increase in peroxynitrite formation in the liver of hyperhomocysteinemic rat. An increase in the level of peroxynitrite in the liver could be due to an elevation of superoxide anion and/or NO levels. Our results clearly demonstrated that activation of NAD(P)H oxidase during hyperhomocysteinemia caused an increase in superoxide anion production in the liver. A significant correlation coefficient value between the serum Hcy level and hepatic superoxide anion content was observed. On the other hand, NOS activity and NO concentration were not altered in the liver during hyperhomocysteinemia. These results suggested that peroxynitrite formation in the liver was mainly due to Hcy-induced superoxide anion production. Second, peroxynitrite is a potent oxidant that can damage cells by modifying lipids, proteins and DNA. There was a significant increase in the levels of lipid peroxides and nitrotyrosine protein adducts in the livers of hyperhomocysteinemic rats, suggesting oxidative damage occurred. Third, the involvement of NAD(P)H oxidase was furthered confirmed by using a known NAD(P)H oxidase inhibitor, apocynin. The NAD(P)H oxidase activity, superoxide anion level, peroxynitrite formation as well as lipid peroxidation were reduced to the basal levels in hyperhomocysteinemic rats that were treated with apocynin. Taken together, these results indicate that NAD(P)H oxidase dependent superoxide anion generation plays a major role in Hcy-induced oxidative stress in liver injury during hyperhomocysteinemia.

Folic acid supplementation is viewed as a promising treatment for patients with cardiovascular disease associated with hyperhomocysteinemia. Oral folic acid supplementation has been shown to improve the endothelium-dependent vascular function in patients with mild hyperhomocysteinemia [19–21]. The mechanisms underlying such an effect are not fully understood. It has been proposed that the beneficial effect of folic acid in cardiovascular disease is attributed to Hcy reduction as well as its ability to antagonize oxidative stress [19–21]. In the present study, folic acid supplementation to rats fed a high-methionine diet resulted in a 2.9-fold increase in the serum folate level as compared to the control group. Such a treatment led to a significant reduction in the serum Hcy level (from 25.48 μM to 18.66 μM). Although the serum Hcy level in rats fed a high-methionine diet plus folic acid was still significantly higher than that found in the control group (18.66 μM versus 3.52 μM), folic acid supplementation completely abolished hyperhomocysteinemia-induced superoxide anion generation, peroxynitrite formation, lipid peroxidation in the liver. Furthermore, folic acid supplementation could alleviate liver injury in hyperhomocysteinemic rats. It is tempting to speculate that the hepatic protective effect of folic acid supplementation may be mediated, in part, via its direct action that is independent of its Hcy lowering effect. On the other hand, it is possible that reduction of serum Hcy levels by folic acid supplementation below a certain threshold may be able to abolish hyperhomocysteinemia-induced oxidative stress and liver injury. Although Hcy at the serum concentration of 18.66 μM did not elicit oxidative stress and liver injury, it remains to be investigated whether such a reduction of serum Hcy levels in rats by folic acid supplementation is sufficient to improve endothelial function. Interestingly, in rats fed 0.8% methionine diet, the serum Hcy level was double of the control value but there was no increase in hepatic lipid peroxidation (Fig. 7). Furthermore, vitamin B_6 supplementation caused Hcy-lowering effect similar to that of folic acid supplementation and effectively abolished Hcy-induced oxidative stress (Fig. 6).

These results suggested that decreasing Hcy to a certain level might be sufficient to prevent hepatic oxidative stress. However, results from the present study could not rule out the possibility of direct effect of folic acid or vitamin B_6 against oxidative stress.

To the best of our knowledge, this is the first study to show that in the absence of other known risk factors, diet-induced hyperhomocysteinemia can cause liver injury and folic acid supplementation offers a hepatoprotective effect. Increased superoxide anion generation may serve as one of the mechanisms for Hcy-induced oxidative stress and liver injury. Our results suggest that the role of folic acid in maintaining good health may extend beyond the cardiovascular system to encompass the hyperhomocysteinemia-associated disorders that involve other organs such as liver.

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