

A nutrient mixture suppresses carbon tetrachloride-induced acute hepatic toxicity in ICR mice

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We examined the effect of a nutrient mixture (NM) that contains lysine, proline, ascorbic acid, and green tea extract in mice treated with carbon tetrachloride (CCl₄), a model of liver injury in which free radical, oxidative stress, and cytokine production are closely linked. Seven-week-old male ICR mice were divided into four groups (A–D) of five animals each. Groups A and C mice were fed a regular diet for 2 weeks, whereas groups B and D mice were supplemented with 0.5% NM (w/w) during that period. Groups A and B received corn oil i.p., whereas groups C and D received CCl₄ (25 µL/kg, in corn oil, i.p.). All animals were killed 24 h after CCl₄ administration, serum was collected to assess liver and kidney functions, and livers and kidneys were excised for histology. Mean serum aspartate aminotransferase

and alanine aminotransferase were comparable in groups A and B, increased markedly in group C, and significantly lowered in group D compared with group C. CCl₄ had no significant effect on renal markers (blood urea nitrogen [BUN], creatinine, and BUN/creatinine ratio). CCl₄ administration caused an intense degree of liver necrosis that was less severe in the NM fed group D. These results indicate that NM could be a useful supplement in preventing acute chemical-induced liver toxicity.

Key words:

Introduction

Oxygen-derived free radicals and lipid peroxidation play a critical role in the pathogenesis of various liver diseases including hepatic fibrosis.^{1,2} Accordingly, elimination of free radicals and prevention of lipid peroxidation have been targeted in prevention and treatment of hepatic damage. Carbon tetrachloride (CCl₄), a classic hepatotoxicant that causes acute liver injury, has been used to study liver pathology associated with free radical oxidative stress and cytokines.^{3,4} CCl₄-induced hepatotoxicity involves two phases: the initial phase during which toxic chemicals initiate injury modulated by the net effect of bioactivation and detoxification processes and the subsequent phase when progression or regression of the injury occurs, secondary to the absence or presence of compensatory tissue repair.⁵ During metabolism of CCl₄ by the mitochondrial monooxygenase (P450 2E1) system, an unstable trichloromethyl peroxide (CCl₃) free radical is formed, and rapidly converted to trichloromethyl peroxide

(Cl₃COO⁻).⁶ These radicals lead to peroxidation of fatty acids found in phospholipids making up the cell membranes. Lipid peroxide radicals, lipid hydroperoxides, and lipid breakdown products develop, leading to activation of Kupffer cells by free radicals accompanied by production of proinflammatory mediators.^{3,7}

Despite the elimination of CCl₄ within 24 h, because of its volatility, liver injury progresses. Limaye, *et al.*⁸ found that calpain released from dying hepatocytes mediated progression of acute liver injury induced by CCl₄. Bhawe, *et al.*⁹ hypothesized that injury progresses secondary to extracellular appearance of hydrolytic enzymes, such as secretory phospholipase A₂ (sPLA(2)) following leakage or upon cell lyses in the absence of sufficient cyclooxygenase-2 (COX-2). In addition, sPLA(2), secreted for cleanup of necrotic debris upon initiation of hepatic necrosis, required the induction of sufficient COX-2 to prevent the unchecked destructive action of sPLA(2).¹⁰

Diverse antioxidants have been shown to prevent CCl₄-induced hepatotoxicity in rats.^{11–17} A unique nutrient formulation (NM) containing primarily of ascorbic acid, lysine, proline, N-acetyl cysteine, and green tea extract has previously been shown to exhibit

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a broad spectrum of pharmacological, therapeutic, cardiovascular, and chemoprotective properties.^{18–21} In a recent study, we found that NM reported significant inhibition of acetaminophen-induced hepatic and renal damage, including histopathology and hepatic and renal serum enzyme levels.²² In the current study, we examined the in-vivo effects of the nutrient mixture (NM) in mice treated with CCl₄, a model of liver injury in which free radical, oxidative stress, and cytokine production are closely linked.

Methods and materials

Materials

CCl₄ (density of 1.594 g/mL) was obtained from Sigma Chemical Co. (St Louis, Missouri, USA) and diluted in corn oil 1:10 (v/v). Stock solution of the NM was composed of the following in the ratio indicated: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 µg; copper 2 mg; manganese 1 mg.

Animals

Male ICR mice, free of murine viruses, bacteria, and parasites, approximately 6 weeks of age on arrival, were purchased from Simonsen Laboratories (Gilroy, California, USA) and maintained in microisolator cages under pathogen-free conditions on a 12-h light/12-h dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals.

Experimental design

After 1 week of isolation, 7-week-old male ICR mice ($n = 20$) weighing 30–32 g were divided into four groups of five mice each (A–D). Groups A and C mice were fed a regular mouse chow diet for 2 weeks, whereas groups B and D mice were fed the regular diet supplemented with 0.5% NM (w/w) during that period. The two diets are isocaloric. Although the quantity of diet provided to the mice was unrestricted, the mice consumed, on the average, 4 g of their respective diets per day. Thus, the supplemented mice received approximately 20 mg of NM per day, which indicates that they received the following amounts of NM components per day: ascorbic acid 3.5 mg, L-lysine 5 mg, green tea extract 5 mg, L-proline 3.75 mg, L-arginine 2.5 mg, N-acetyl cysteine 1 mg, selenium 0.15 µg, copper 0.01 mg, manganese 5 µg. Dietary pretreat-

ment for 2 weeks was used to prime the mice with nutrients before challenge with CCl₄ injection.

Subsequently, corn oil was administered i.p. to group A mice (control) and group B mice; CCl₄ (25 µL/kg body weight) was administered i.p. to groups C and D. Mice received 0.2 mL/20 g body weight of stock solution of CCl₄ (25 µL in 10 mL of corn oil). The dose of CCl₄ administered was based on the work of Chen, *et al.*¹⁵ in which the lowest dose of CCl₄ that produced histologic evidence of liver necrosis was determined using ICR mice. That dose was 20 µL/kg. All animals were killed 24 h after CCl₄ or corn oil administration. Mice were anesthetized by inhalation with isoflurane USP (Abbott Labs, Chicago, Illinois, USA), the abdominal cavity was opened, and approximately 1 mL of blood was removed by cardiac puncture from each mouse. Blood was allowed to clot and blood samples were spun at 3000 rpm for 5 min at 4 °C. The samples were stored at –80° until they were sent for renal and hepatic enzyme analysis. Kidneys and livers were excised from the mice, weighed, and processed for histology.

Histology

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4–5 microns. Sections were deparaffinized through xylene and graduated alcohol series to water and stained with hematoxylin and eosin (H & E) for evaluation using a standard light microscope.

Serum analyses

All chemistry tests for liver: alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP), and kidney: creatinine and blood urea nitrogen (BUN) functions were run on a Hitachi 747 Chemistry Analyzer with Boehringer Mannheim Corporation reagents.

Statistical analysis

The results were expressed as means ± SD for the groups. Data were analyzed by one-way ANOVA. The independent sample “*t*” test was used to compare results for the two CCl₄-injected groups, as well as to independently compare the negative and positive control groups.

Results

Mean body weights

Mean body weights of mice did not differ significantly between groups ($P = 0.63$). Initial body

weights of mice ranged between 30 and 32 g; on killing, mean body weights of groups were: A (control) 30.9 ± 2.5 g; B (NM) 32.2 ± 1.7 g, C (CCl_4) 27.9 ± 2.3 g, and D (CCl_4 + NM) 31.6 ± 1.0 g.

Liver and kidney weights

Mean liver weight of ICR mice increased by 4% over control liver weight with CCl_4 administration (1.93 ± 0.26 g for group C and 2.1 ± 0.38 g for group D), but did not reach statistical significance ($P = 0.60$). Mean liver weights of control group A and NM-supplemented group B were 1.86 ± 0.13 g. CCl_4 treatment resulted in 19% increase in mean kidney weight of ICR mice over control kidney weight, but did not reach statistical significance ($P = 0.16$). Mean kidney weights of control group A and NM group B were 0.52 ± 0.05 g and 0.50 ± 0.06 g, respectively. Mean kidney weights of CCl_4 -treated groups were 0.62 ± 0.14 g for group C and 0.57 ± 0.25 g for group D.

Liver histology and gross appearance

Although gross analysis of livers from all mice showed no apparent abnormality, histological evaluation showed significant differences among the groups. CCl_4 caused significant centrilobular necrosis in livers of unsupplemented (group C animals), as shown in Figure 1C, whereas dietary supplementation with 0.5% NM before CCl_4 treatment reduced the extent of these alterations in group D mice (Figure 1D). Control (group A) and NM 0.5% diet supplemented (group B) mice that received corn oil instead of CCl_4 injections reported liver histology within normal limits, as shown in Figure 1A and B, respectively.

Renal histology and gross appearance

Gross analysis of kidneys from all mice as well as histological evaluation showed no apparent abnormalities (photos not shown).

Liver serum markers

ALT and AST levels in ICR mice not treated with CCl_4 (control group A and NM supplemented group B) were comparable, significantly elevated in CCl_4 -treated mice (group C), and significantly reduced in mice supplemented with NM before CCl_4 injection. AP was not significantly affected by CCl_4 administration. NM supplementation, with and without CCl_4 challenge, reduced serum level of AP. See Table 1 for serum ALT, AST, and AP values. ANOVA one way variance analysis reported that serum ALT, AST, and AP results reached statistical

significance of $P < 0.001$, $P = 0.001$, and $P = 0.014$, respectively.

Serum ALT values for groups A and B were 25 ± 3 and 28 ± 2 IU/L. Serum ALT was increased to $19,680 \pm 1640$ IU/L (78,720% of control) with CCl_4 administration, as shown in Figure 2A. Pretreatment of mice with the NM diet before CCl_4 reduced the effect of CCl_4 by 25% ($P = 0.004$) to $14,841 \pm 2105$ IU/L. Serum AST for groups A and B were 55 ± 8 and 76 ± 8 IU/L, respectively. Serum AST increased to 7900 ± 1800 IU/L (14,364% of control) with CCl_4 administration, as shown in Figure 2B. Pretreatment of mice with the NM diet before CCl_4 administration reduced the effect of CCl_4 by 42% ($P < 0.0001$) to 4635 ± 980 IU/L. Serum AP for groups A and B were 178 ± 10 and 112 ± 8 IU/L, respectively. Serum AP was not significantly affected by CCl_4 administration, as shown in Figure 2C. Treatment of mice with the NM diet without CCl_4 challenge reduced serum level of AP by 38% to 112 ± 8 IU/L ($P < 0.0001$) of the untreated control and CCl_4 challenged mice by 33% to 128 ± 8 IU/L ($P < 0.0001$).

Renal serum markers

Serum markers of kidney: BUN, creatinine, and BUN/creatinine ratio were not significantly affected by CCl_4 treatment, as shown in Table 2. Serum BUN values for groups A–D were 22.2 ± 0.5 mg/dL, 17 ± 4 mg/dL, 17.2 ± 1.7 mg/dL, and 19 ± 2 mg/dL, respectively. Serum creatinine values for groups A–D were 0.52 ± 0.04 mg/dL, 0.48 ± 0.06 mg/dL, 0.46 ± 0.02 mg/dL, and 0.44 ± 0.02 mg/dL, respectively. Serum BUN/creatinine ratios for groups A–D were 44 ± 4 , 47 ± 5 , 38 ± 0.5 , and 44 ± 0.5 .

Discussion

CCl_4 treatment caused significant hepatic centrilobular necrosis and marked increases in hepatic serum markers AST and ALT in unsupplemented mice. Table 3 summarizes the correlations between liver histology and liver functional enzyme levels in all groups. Elevated serum ALT and AST levels reflect enzyme leakage from damaged or necrotic hepatocytes. NM reduced AP levels in both CCl_4 treated and untreated mice for reasons unexplored. However, the AP levels in the mice treated with NM alone (group B) were still within normal limits. Furthermore, low AP levels have no clinical relevance. However, high levels of AP are associated with biliary tract damage and inflammation.

The results report that pretreatment of ICR mice with a diet supplemented with 0.5% of the NM for

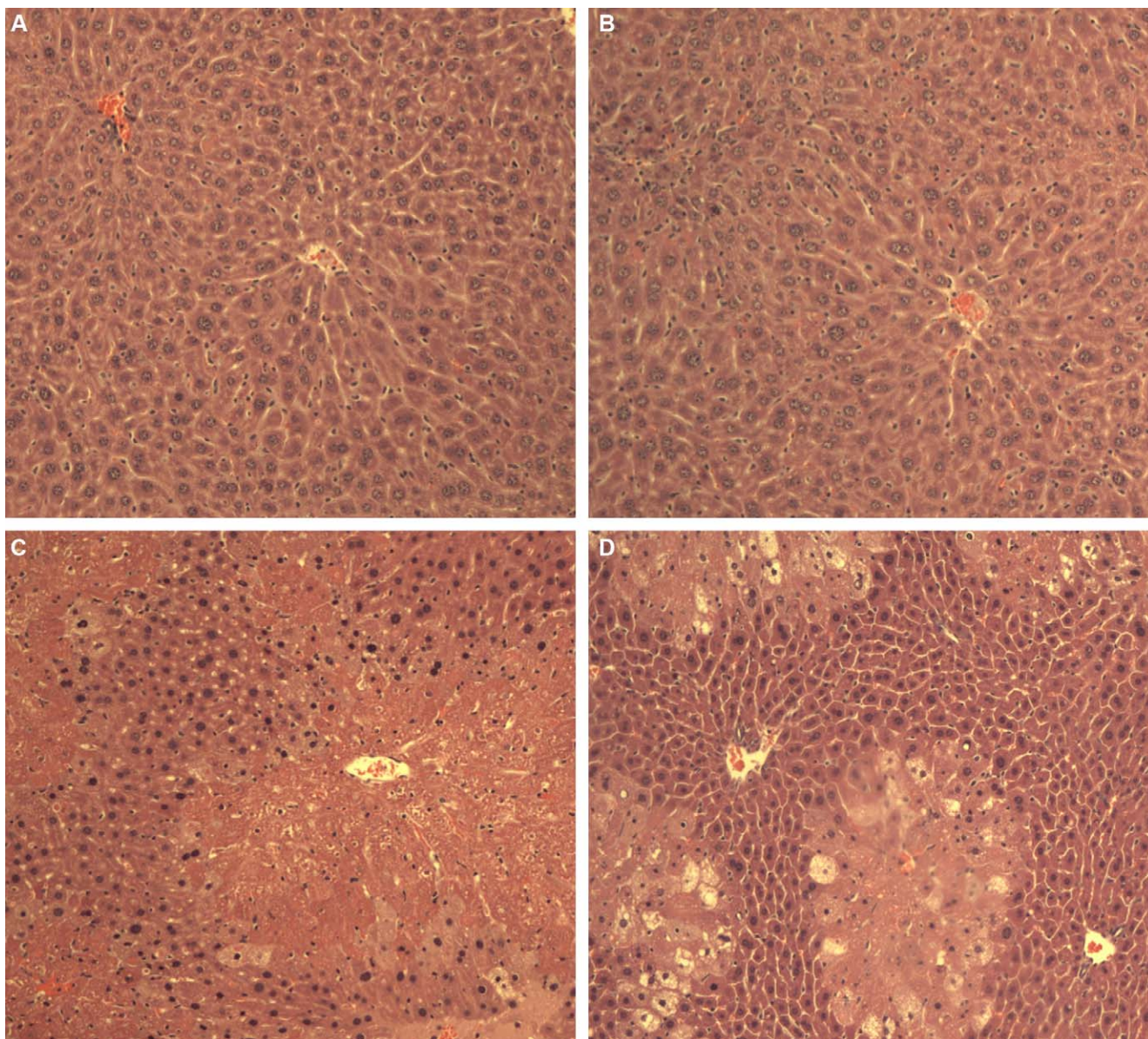


Figure 1 (A) Sample liver section from untreated (control group A) ICR mice H&E 200 \times . Liver section within normal limits. (B) Sample liver section from NM supplemented (group B) ICR mice H&E 200 \times . Liver section within normal limits. (C) Sample liver section from ICR mice treated with CCl₄ 25 μ L/kg (group C) H&E 200 \times . CCl₄ caused significant centrilobular necrosis in unsupplemented mice. (D) Sample liver section from NM 0.5% supplemented ICR mice treated with CCl₄ μ L/kg 200 \times (group D). NM treatment provided some protection; liver section shows mild-to-moderate centrilobular necrosis.

2 weeks reduced hepatic damage in ICR mice that also received a toxic dose of CCl₄. Supplementation with NM reduced hepatocellular necrosis as well as the serum AST, ALT, and AP levels.

In acute toxicity, absence or presence of tissue repair responses lead to either progression or regression of injury, respectively. Various factors have been shown to affect tissue repair. Newborn animals (under 2 months of age) are capable of mounting faster and efficient tissue repair than adults. In addition, diet restriction has been shown to protect from

chemical-induced toxicity⁹. In our study, the animals were all 7 weeks of age, and thus equally not as efficient at mounting efficient tissue repair. Furthermore, the diets provided were equicaloric and unrestricted, and thus not a factor.

The NM tested was formulated based on targeting different physiological processes involved in a wide spectrum of pathological conditions at the cellular level. Several research teams have reported that much of CCl₄-induced liver damage may be due to increased oxidative damage and the appearance of

Table 1 Mean \pm SD values of liver functional enzymes among ICR mouse groups

Groups	<i>CCl₄ or corn oil injection</i>	<i>2-week diet</i>	<i>ALT (IU/L)</i>	<i>AST (IU/L)</i>	<i>AP (IU/L)</i>
A (n = 5)	Corn oil	Regular mouse chow	25 \pm 3	55 \pm 8	178 \pm 10
B (n = 5)	Corn oil	0.5% NM supplemented	28 \pm 2	76 \pm 8	112 \pm 8
C (n = 5)	CCl ₄ 25 μ L/kg	Regular mouse chow	19,680 \pm 1,640	7,900 \pm 1,800	190 \pm 18
D (n = 5)	CCl ₄ 25 μ L/kg	0.5% NM supplemented	14,841 \pm 2,105	4,635 \pm 980	128 \pm 6

ANOVA one-way variance analysis for ALT, AST, and alkaline phosphatase reached significance levels of $P < 0.001$, $P = 0.001$, and $P = 0.014$, respectively.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphatase; NM, nutrient formulation.

increased free radicals. Diverse antioxidants have been shown to prevent CCl₄-induced hepatotoxicity in animal models. Ademuyiwa, *et al.*¹³ reported that vitamin C prevented CCl₄-induced liver damage in rats. Repeated doses of vitamins C and E were shown to provide a pronounced protective effect against toxin-induced liver enzyme elevation in rats.¹¹ Sun, *et al.*¹⁴ showed that CCl₄ consumes vitamin C, especially in the liver; liver concentration of vitamin C was decreased significantly within 24 h of CCl₄ administration, although the level of vitamin C in the plasma was not significantly affected. Gonskii, *et al.*²³ also showed that CCl₄ lowered vitamin C levels as well as antioxidant levels in the liver.

Green tea polyphenols have also been shown to prevent liver injury from CCl₄-induced hepatotoxicity.^{15,16} Pretreatment with EGCG led to a dose-dependent decrease in all of the histological and biochemical variables of liver injury observed in the CCl₄-treated (20 μ L CCl₄/kg weight) mice. Green tea polyphenols reduced the severity of liver injury with lower concentrations of lipid peroxidation and proinflammatory nitric oxide generated mediators. Hasegawa, *et al.*²⁴ reported pretreatment of male rats with 2% green tea supplemented drinking water for 2 weeks before a single i.p. injection of the carcinogen 2-nitropropane (2NP) at a dose of 100 mg/kg body weight of rats provided effective protection against induction of hepatic degenerative changes by 2NP. Green tea effectively blocked oxidative DNA damage to the liver as well as hepatotoxicity in rats treated with 2NP.

Another component of the NM important for protection of the liver from CCl₄ toxicity is *N*-acetyl cysteine, which acts by increasing glutathione stores and as an antioxidant inhibiting neutrophil accumulation. Maksimchik, *et al.*²⁵ reported that NAC administration (3 \times at 150 mg/kg) to rats treated with CCl₄ (4 g/kg) prevented oxidative damage of liver cells, decreased membrane lipid peroxidation, protein carbonyls and mixed protein-glutathione disulphide formation and partially normalized plasma triacylglycerols, although it did not effect a

marked decrease in the elevated blood ALT and AST levels.

The role of matrix metalloproteinases (MMPs) in CCl₄-induced hepatic centrilobular necrosis was reported by Jiang, *et al.*²⁶ The research group reported that CCl₄-induced pathology from 4-week administration of the toxin to mice was reversed after 4 weeks of withdrawal from CCl₄ treatment except for the induced fibrotic changes. One of the most significant changes caused by CCl₄ treatment was a remarkably increased expression of extracellular matrix genes. Unlike other genes, the up-regulation of these genes, including procollagen, MMPs, integrins, among others, remained high after 4 weeks cessation of CCl₄ treatment. Zhen, *et al.*¹⁶ reported that daily i.p. injections of EGCG prevented CCl₄-induced hepatic fibrosis and MMP-2 activity of EGCG-treated hepatic stellate cells. Our previous studies with a wide variety of cell types have shown that NM inhibits MMP-2 and MMP-9 secretion and cellular invasion through the ECM.^{18,20} In addition, we have previously shown that the ECM synthesized by normal fibroblasts treated with NM for 7 days reported increased stability to invasion by tumor cells (by changes in the ECM composition) and significantly reduced MMP-2 and MMP-9 secretion and adhesion to collagen I and other substrates.²⁷

ECM integrity is dependent upon adequate collagen formation and its stability. In this aspect, ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers.²⁸ Manganese and copper are also essential cofactors in collagen formation process. Collagen stability can be controlled by lysine²⁸ and also by *N*-acetyl cysteine through its inhibitory effect on MMP-9 activity²⁹ as well as migration of endothelial cells through ECM.³⁰ Selenium has been shown to interfere with MMP expression and tumor invasion.³¹ Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression

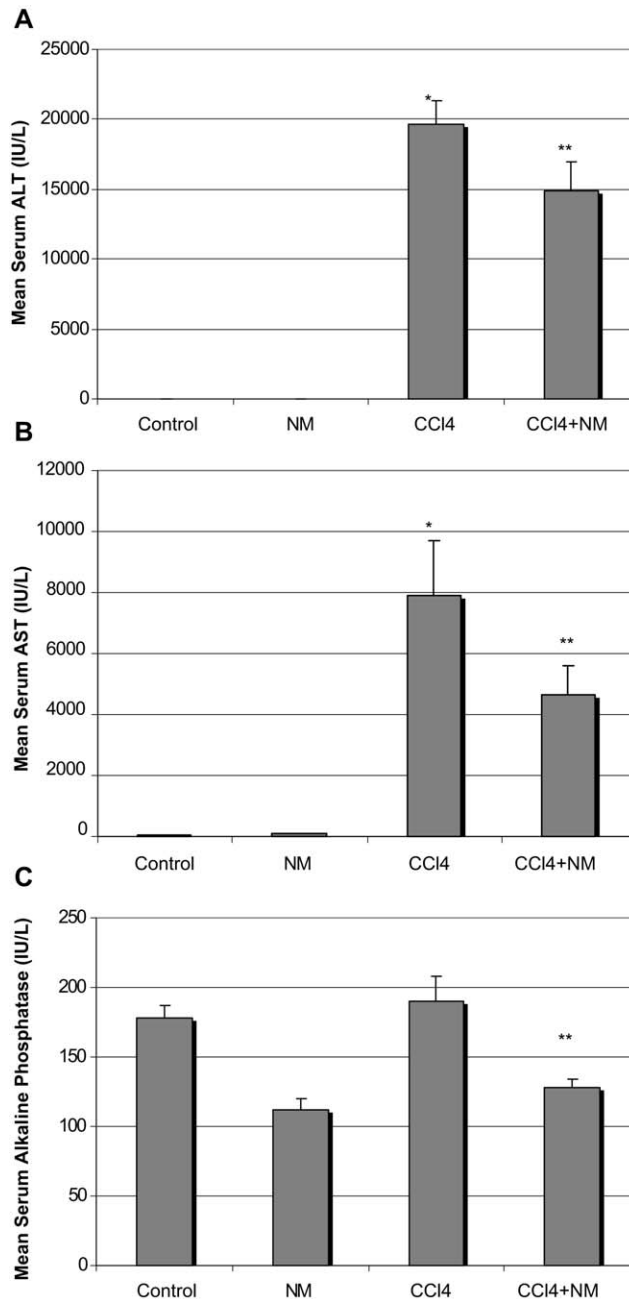


Figure 2 (A) Effect of CCl₄ administered to NM-supplemented and unsupplemented ICR mice on serum alanine aminotransferase (ALT). Effect of CCl₄ (25 μL/kg) administered to NM-supplemented and unsupplemented ICR mice on serum ALT. Serum ALT in ICR mice not treated with CCl₄ (control group A and NM supplemented group B) were comparable. Serum ALT was increased to 19,680 ± 1640 IU/L (78,720% of control) with CCl₄ administration. Supplementation with NM before CCl₄ injection (group D) reduced the effect of CCl₄ by 25% ($P = 0.004$). * $P < 0.0001$ with respect to the untreated control (group A); ** $P = 0.004$ with respect to CCl₄-treated control (group C). (B) Effect of CCl₄ (25 μL/kg) administered to NM-supplemented and unsupplemented ICR mice on serum aspartate aminotransferase (AST). Serum AST in ICR mice not treated with CCl₄ (control group A and NM-supplemented group B) were 55 ± 8 and 76 ± 8 IU/L, respectively. Serum AST was increased to 7900 ± 1800 IU/L (14,364% of control) with CCl₄ administration. Treatment of mice with the NM diet before CCl₄ reduced the effect of CCl₄ by 42% ($P < 0.0001$) to 4635 ± 980 IU/L. * $P < 0.0001$ with respect to the untreated control (group A); ** $P < 0.0001$ with respect to CCl₄-treated control (group C). (C) Effect of CCl₄ (25 μL/kg) administered to NM-supplemented and unsupplemented ICR mice on serum alkaline phosphatase (AP). AP in ICR mice not treated with CCl₄ (control group A and NM supplemented group B) were comparable. Treatment of mice with the NM diet without CCl₄ challenge reduces serum level of alkaline phosphatase by 38% ($P < 0.0001$) of the untreated control and CCl₄-challenged mice by 33% to 128 ± 8 IU ($P < 0.0001$). * $P < 0.0001$ with respect to untreated control (group A); ** $P = 0.0001$ with respect to CCl₄-treated control (group C).

Table 2 Mean \pm SD values of renal functional enzymes among ICR mouse groups

Groups	<i>CCL</i> ₄ or corn oil injection	2-week diet	BUN (mg/dL)	Creatinine (mg/dL)	BUN/creatinine
A (n = 5)	Corn oil	Regular mouse chow	22.2 \pm 0.5	0.52 \pm 0.04	44 \pm 4
B (n = 5)	Corn oil	0.5% NM supplemented	17 \pm 4	0.48 \pm 0.06	47 \pm 5
C (n = 5)	<i>CCL</i> ₄ 25 μ L/kg	Regular mouse chow	17.2 \pm 1.7	0.46 \pm 0.02	38 \pm 5
D (n = 5)	<i>CCL</i> ₄ 25 μ L/kg	0.5% NM supplemented	19 \pm 2	0.44 \pm 0.02	44 \pm 5.4

ANOVA one-way variance analysis for BUN reached significance level of $P = 0.048$; ANOVA analysis for creatinine ($P = 0.523$), and BUN/creatinine ($P = 0.646$) did not reach statistical significance. BUN, blood urea nitrogen; NM, nutrient formulation.

Table 3 Correlation between liver histology and liver functional enzymes among ICR mouse groups

Group	Liver histology	Liver enzymes (ALT AST AP)
A – corn oil injection and regular diet	Within normal limits	+ + +
B – corn oil injection and NM 0.5% diet	Within normal limits	+ + +
C – <i>CCL</i> ₄ injection and regular diet	Severe centrilobular necrosis	++++ +++ +
D – <i>CCL</i> ₄ injection and NM 0.5% diet	Mild-to-moderate centrilobular necrosis	+++ ++ +

Liver histology correlated with ALT and AST values. *CCL*₄ administration caused severe centrilobular necrosis and associated large increases in ALT and AST serum levels. Dietary pretreatment with NM 0.5% diet before *CCL*₄ injection resulted in mild-to-moderate centrilobular necrosis and reduced ALT and AST levels. AP remained within normal limits in all groups. ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphatase; NM, nutrient formulation.

through inhibition of MMPs and cellular invasion.³²

On the basis of our own research and published data, we postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, *N*-acetyl cysteine, selenium, copper, and manganese would result from their synergy.^{33,34} For example, we found that a combination of ascorbic acid, lysine, and proline used with EGCG enhanced the anti-invasive activity of 20 μ g/mL EGCG to that of 50 μ g/mL on human fibrosarcoma cells.³³ Thus, by including nutrients such as *N*-acetyl cysteine, arginine, selenium, manganese, and copper in addition to ascorbic acid, proline, lysine, and EGCG, we could obtain significant reduction in fibrosarcoma cell invasion at a much lower concentration of EGCG.

In conclusion, this study reported that pretreatment for 2 weeks with a diet supplemented with 0.5% NM reduced hepatic damage in ICR mice from administration of a toxic dose of *CCL*₄. Supplementation of ICR mice with dietary NM reduced the pathological histological changes in the liver, as well as reversed the *CCL*₄-induced elevated hepatic serum markers.

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