JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Hepatoprotection of Noni Juice against Chronic Alcohol Consumption: Lipid Homeostasis, Antioxidation, Alcohol Clearance, and Anti-inflammation

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ABSTRACT: Chronic alcohol consumption leads to steatohepatitis and cirrhosis. Naturally fermented noni juice (NJ) contains polyphenols, polysaccharides, and some trace minerals. This study explored protective effects of NJ against chronic alcohol consumption. Mice were assigned randomly to one of the following groups: (1) control, control liquid diet and distilled water; (2) alcohol, alcohol liquid diet and distilled water; (3) Alc+NJ_1X, alcohol liquid diet and 5 mL NJ/kg BW; (4) Alc+NJ_2X, alcohol liquid diet and 10 mL NJ/kg BW; (5) Alc+NJ_3X, alcohol and 15 mL NJ/kg BW for 4 weeks. NJ decreased ($p < 0.05$) serum AST, ALT, and alcohol levels and liver lipids, as well as increased ($p < 0.05$) daily fecal lipid outputs in alcohol-diet fed mice. NJ supplementation not only down-regulated ($p < 0.05$) lipogenesis but also up-regulated ($p < 0.05$) fatty acid β-oxidation in livers of alcohol-diet fed mice. NJ also accelerated alcohol clearance via increased ($p < 0.05$) hepatic ADH and ALDH activities. NJ increased ($p < 0.05$) hepatic TEAC and GSH levels but decreased ($p < 0.05$) TBARS value and TLR2/4, P38, ERK 1/2, NFκB P65, iNOS, COX-2, TNF-α, and IL-1β expressions in alcohol-diet fed mice. NJ promotes hepatoprotection against alcohol-induced injury due to regulations of lipid homeostasis, antioxidant status, alcohol metabolism, and anti-inflammatory responses.

KEYWORDS: *alcohol metabolism, anti-inflammatory response, antioxidant capacity, lipid homeostasis, noni juice*

■ INTRODUCTION

Chronic liver disease and cirrhosis are serious diseases endangering to either the worldwide population or Taiwan specifically. Fatty liver is characterized by an accumulation of triacylglycerol in hepatocytes via a steatosis progression. The fatty liver disease is mainly divided as two categories: alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD). It was reported that approximately 80% of heavy drinkers develop steatosis, 35% develop alcoholic hepatitis, and 10% develop cirrhosis, whereas 28% of all deaths are from liver diseases and 40% of those deaths are from cirrhosis.[1] The physiological changes of alcohol-induced liver injury involve enhancement of lipogenesis, inhibition of fatty acid oxidation, formation of reactive oxygen-containing molecules, and production of inflammatory responses that further lead to steatohepatitis and cirrhosis.

The primary enzymes involved in alcohol metabolism include alcohol dehydrogenase (ADH), cytochrome P4502E1 (CYP2E1), and catalase, which catalyze alcohol to acetaldehyde, as well as acetaldehyde dehydrogenase (ALDH), which finally metabolizes acetaldehyde to acetate.[2] However, during alcohol metabolism by CYP2E1 reactive oxygen species (ROS) are also generated, and then lipid peroxidation, that is, malondialdehyde (MDA), is increased in the liver.[3] Meanwhile, an intermediary metabolite, acetaldehyde, also easily produces acetaldehyde−protein adducts, which accelerate production of lipid peroxidation, decrease antioxidants, and deplete the mitochondrial and cytosolic pool of glutathione (GSH), thus further enhancing liver damage.[2] Chronic alcohol consumption increases the $NADH/NAD^+$ ratio, which up-regulates lipogenesis but down-regulates β-oxidation of fatty acids.[4] With regard to the development of hepatic steatosis, increased expressions of sterol regulatory element binding protein-1c (SREBP-1c), acetyl CoA carboxylase (ACC), and fatty acid synthase (FAS) are related to de novo lipogenesis in the liver, whereas decreased expressions of retinoid X receptor alpha (RXR-α), proliferator-activated receptor-alpha (PPAR-α), and uncoupling protein 2 (UCP2) result in hepatic lipid accumulation.[5−7] Besides, mitogen-activated protein kinases (MAPKs) regulate a variety of biologic processes, such as cell growth,

Received: August 4, 2013
Revised: October 22, 2013
Accepted: October 24, 2013
Published: October 24, 2013

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proliferation, and inflammation. It was indicated that alcohol activates MAPK pathways, which respond to extracellular stimuli via targeting transcription factors and modulating gene expressions, such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and P38 MAPK.[8] Toll-like receptor 4 (TLR4) is a key component involved in innate immune system activation and stimulates nuclear factor kappa B (NFκB) and tumor necrosis factor-alpha (TNF-α) in alcohol-induced liver injury.[9] The activation of NFκB also accelerates inflammatory mediator expressions, such as inducible nitric oxide synthase (iNOS) and cyclooxygenease-2 (COX-2), and creates a cycle of inflammatory responses.[10]

Noni (*Morinda citrifolia*) belongs to the family Rubiaceae (coffee family), subfamily Rubioideae, and has been locally planted in southern Taiwan for decades. Polysaccharides, fatty acid ester, glycosides, iridoids, anthraquinones, flavonoids, phytosterols, carotenoids, vitamin A, antraquinones, potassium, etc., have been identified as putative active ingredients in noni juice.[11−13] Previous studies also indicated that these bioactive ingredients impart anti-inflammatiory effects via regulating secretions of interleukin-1 beta (IL-1β), IL-6, and PGE_2,[14] as well as antitumorigenic effects via suppressing cell transformation and blocking of phosphorylation of c-Jun.[15] In our previous studies, phenolic acids and polysaccharides were major bioactive components in fermented noni juice (NJ).[16,17] Moreover, NJ showed hypolipidemic, antioxidative, and anti-inflammatory effects in high-fat/cholesterol-diet fed hamsters. However, on the basis of our knowledge, the molecular mechanism of NJ hepatoprotection against chronic alcohol consumption is still lacking. Therefore, by employing a chronic alcohol consumption mouse model, the present study addressed the protective effects of NJ on livers via (1) normalization of lipid homeostasis, (2) increased antioxidative capacities, (3) acceleration of alcohol metabolism, and (4) down-regulation of inflammatory responses.

MATERIALS AND METHODS

Noni Juice Preparation. Noni juice was prepared according to our previous methods.[16,17] Noni fruits were purchased from a local fruit farm (Xuejia District, Tainan City, Taiwan), stored in a stainless steel bottle at room temperature for 1 year, and then separated through a wire mesh screen. The experimental NJ materials were further centrifuged from collected NJ at 3000*g* for 15 min, pasteurized at 80 °C for 60 s, and then stored at −20 °C until used to feed animals.

Phytochemicals and Mineral Analysis of NJ. On the basis of our previous studies,[16,17] major identified phenolic acids in NJ were gallic acid, gentisic acid, chlorogenic acid, *p*-hydroxybezoic acid, caffeic acid, ferulic acid, and *p*-anisic acid, whereas flavonoids in NJ included epicatechin, hesperidin, and naringin. Meanwhile, the amount of phenolic acids in NJ was almost 6 times higher than that of the flavonoids. Gentisic acid, *p*-hydroxybenzoic acid, and chlorogenic acid were the dominant phenolic acids in NJ. Hence, the total amounts of phenol, flavonoid, and condensed tannin contents were measured in this study. Total phenol contents were measured using Folin−Ciocalteu's phenol reagent and expressed as milligrams of gallic acid equivalent (GAE) per 100 mL of sample.[18] Flavonoid contents were determined with 10% $AlCl_3{\cdot}H_2O$ solution and expressed as milligrams of catechin equivalent (CE) per 100 mL of sample.[19] Condensed tannin contents were surveyed through a modified method and expressed as milligrams of CE per 100 mL of sample.[18] Ascorbic acid was quantified according to previous method.[20] The crude polysaccharides were precipitated with 95% ethanol at 4 °C for 12 h.[21] The concentration of total polysaccharide contents in NJ was measured by the absorbance at 490 nm (UV−visible spectrophotometer, model T60, PG Instruments Ltd., Leicestershire, UK) against a standard curve (glucose as a standard) by using the phenol−sulfuric method. The metal contents in NJ were designed to mimic extraction using nitric acid. Samples were made up to 25 mL with ddH_2O prior to final analysis by an Optima 2100 DV ICP system (Perkin-Elmer, Shelton, CT, USA).

Animals and Diets. The animal use and protocol were reviewed and approved by the National Taiwan University Care Committee (IACUC no. 100-101). Sixty male C57BL/6J (*B6*) mice of 8 weeks of age were purchased from the BioLASCO Taiwan Co., Ltd., Taipei, Taiwan. Four mice were housed in each cage in an animal room at 22 ± 2 °C with a 12/12 h light/dark cycle. To induce chronic alcoholic liver disease development, mice were fed a Lieber-DeCarli regular EtOH with 5% (v/v) alcohol (35% alcohol-derived calories).[22] After 1 week of acclimation, 60 mice were randomly divided into five groups: (1) control, control liquid diet and distilled water (oral gavage); (2) alcohol, Lieber-DeCarli regular EtOH diet and distilled water (oral gavage); (3) Alc+NJ_1X, Lieber-DeCarli regular EtOH diet and 5 mL NJ (including 107.08 mg of crude polysaccharides)/kg BW (oral gavage); (4) Alc+NJ_2X, Lieber-DeCarli regular EtOH diet and 10 mL NJ (including 214.15 mg of crude polysaccharides)/kg BW; (5) Alc+NJ_3X, Lieber-DeCarli regular EtOH diet and 15 mL NJ (including 321.22 mg of crude polysaccharides)/kg BW for 4 weeks. Mice were sacrificed by CO_2 asphyxiation. Blood samples and heart, liver, kidney, and visceral tissues in the abdominal cavity were removed after sacrifice and stored at −80 °C for further analyses.

Determination of Serum Biochemical Values, Serum Alcohol Levels, Hepatic Lipids, and Fecal Lipids/Bile Acids. The serum biochemical values, that is, triacylglycerol, cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were determined by using commercial enzymatic kits with the SPOTCHEM EZ SP-4430 automated analyzer (ARKRAY Inc., Kyoto, Japan). Serum alcohol level was detected by using a commercial enzymatic kit with an automated analyzer (AU2700, Olympus, First Chemical Ltd., Tokyo, Japan). Hepatic and fecal triacylglycerol and total cholesterol and fecal bile acids were measured by using commercial kits (Randox Laboratories Ltd., Antrim, UK) according to modified methods.[16]

Preparation of Liver Homogenate. The liver homogenate (10%, w/v) was made with phosphate buffer saline (PBS, pH 7.0, containing 0.25 M sucrose), and the supernatant was collected by centrifugation at 12000*g* for 30 min. The protein content in the supernatant was measured according to the procedures of a Bio-Rad protein assay kit (catalog no. 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Determination of Liver Lipid Peroxidation Level and Antioxidant Capacity. The liver thiobarbituric acid reactive substances (TBARS) level, an indicator, was used to determine liver lipid peroxidation, whereas glutathione (GSH), trolox equivalent antioxidant capacity (TEAC), and activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were assayed as indices for liver antioxidant capacities. These assays were performed according to previously described procedures.[23] The measurement of hepatic TBARS value was based on the production of MDA. The liver TBARS value was calculated by taking the extinction coefficient of MDA to be $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nanomoles per milligram of protein. Due to the unique thiol compound in GSH, 2,2-dithiobisnitrobenzoic acid (DTNB) is commonly used for thiol assay. The hepatic GSH content was calculated by taking the extinction coefficient of 2-nitro-5-thiobenzoic acid (NTB) to be $1.36 \times 10^4\ M^{-1}\ cm^{-1}$ at 412 nm and expressed as micromoles of MDA equivalents per milligram of protein. Hepatic GSH-Px activity was measured by taking the extinction coefficient of NADPH to be $6.22 \times 10^6\ nM^{-1}\ cm^{-1}$ at 340 nm and expressed as nanomoles of NADPH oxidized per minute per milligram of protein. Hepatic TEAC was measured by the scavenging 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^+$) capacity, was absorbed at 734 nm, and was expressed as millimoles per milligram of protein. Hepatic SOD was detected by the inhibitory effect of SOD on purpurogallin of pyrogallol oxidation product, was recorded at 420 nm, and was expressed as milliunits per milligram of protein. Hepatic CAT activity was calculated by taking the extinction coefficient of H_2O_2 to be $39.5\ M^{-1}\ cm^{-1}$ at 240 nm and expressed as units per milligram of protein.

Determination of Hepatic TNF-α and IL-1β Levels. Liver TNF-α and IL-1β concentrations were assayed by using ELISA kits based on anti-mouse TNF-α and IL-1β monoclonal antibodies (BD Pharmingen, San Diego, CA, USA) and converted to the TNF-α and IL-1β levels expressed as picograms per milligrams of protein by using standard curves.

RNA Preparation and Quantitative Real-Time PCR. Total RNA was isolated from the stored frozen liver tissues by using the protocol described by EZNA Tissue RNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA). Reverse transcription was carried out with 2 μg of total RNA, 10 μL of reaction buffer, 1 μL of dNTPs, 2.5 μL of oligo-dT (10 μmol/μL), and 200U RTase (Promega, Madison, WI, USA) with diethyl pyrocarbonate (DEPC) H_2O in a final volume of 50 μL at 42 °C for 1 h. After heat inactivation, 1 μL of cDNA product was used for real-time PCR amplification. The appropriate primers of target genes were designed for 18s (GenBank no. NR_003278.2), SREBP-1c (GenBank no. NM_001244003.1), LXR-α (GenBank no. EU007910.1), ACC (GenBank no. NM_133904.2), FAS (GenBank no. NM_007988.3), DGAT (GenBank no. NM_026384.3), RXR-α (GenBank no. NM_011305.3), PPAR-α (GenBank no. NM_001113418.1), UCP2 (GenBank no. NM_011671.4), ADH (GenBank no. M11307.1), ALDH (GenBank no. NM_009656.3), CYP2E1 (GenBank no. L11650.1), TLR2 (GenBank no. NM_011905.3), and TLR4 (GenBank no. NM_021297.2) as follows: 18s sense 5′-CGGTTCTATTTTGTTGGTTTTCG-3′, antisense 5′-GCGCCGGTCCAAGAATT-3′; SREBP-1c sense 5′-AGCTGATGGAGACAGGGAGTTCT-3′, antisense 5′-GGTGGTGGAAGCCATGCT-3′; LXR-α sense 5′-GCTCTGCTCATAGCCATCAG-3′, antisense 5′-CAGGGCCTCCACATATGTGT-3′; ACC sense 5′-GCAGGTCCAGTTTCTTGTGTT-3′, antisense 5′-TTCCAGATGCTAATGGGTTG-3′; FAS sense 5′-CCTGGATAGCATTCCGAACCT-3′, antisense 5′-AGCACATCTCGAAGGCTACACA-3′; DGAT sense 5′-AGTGGCAATGCTATCATCATCGT-3′, antisense 5′-AAGGAATAAGTGGGAACCCAGATCA-3′; RXR-α sense 5′-CGCAAAGACCTGACCTACACC-3′, antisense 5′-TCCTCCTGCACAGCTTCCC-3′; PPAR-α sense 5′-TGACACCTTCCTCTTCCCAAA-3′, antisense 5′-CGTCGGACTCGGTCTTCTTG-3′; UCP2 sense 5′-TCCCTTGCCACTTCACTTCT-3′, antisense 5′-GCTGCTCATAGGTGACAAACA-3′; ADH sense 5′-GGCCGCCTTGACACCAT-3′, antisense 5′-GCACTCCTACGACGACGCTTA-3′; ALDH sense 5′-CGAACGTCTGCCCTATCAACTT-3′, antisense 5′-CCGGAATCGAACCCTGATT-3′; CYP2E1 sense 5′-AACAGAGACCACCAGCACA-3′, antisense 5′-GGAAGGGACGAGGTTGATGA-3′; TLR2 sense 5′-GGCAGTCTTGAACATTTGGATTT-3′, antisense 5′-CCCCAGTGTCTGGTAAGGATTTC-3′; TLR4 sense 5′-ATGAGGACTGGGTGAGAAATGAG-3′, antisense 5′-GCAATGGCTACACCAGGAATAAAG-3′. The size of reaction products is as follows: 18s, 101 bp; SREBP-1c, 124 bp; LXR-α, 100 bp; ACC, 336 bp; FAS, 122 bp; DGAT, 150 bp; RXR-α, 135 bp; PPAR-α, 107 bp; UCP2, 240 bp; ADH, 76 bp; ALDH, 76 bp; CYP2E1, 199 bp; TLR2, 123 bp; TLR4, 106 bp. Real-time PCR analysis with SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) was performed on a StepOne Real-Time PCR System (Applied Biosystems,).

Western Blot Immunoassay. The liver CYP2E1, NFκB P65, iNOS, COX-2, JNK, P38, and ERK1/2 protein expressions in each group were determined by using a Western blotting method. The clear liver-homogenate supernatant was denatured for 5 min in boiling water with sample buffer (50 mM Tris-HCl, 40 mM β-mercaptoethanol, 2% SDS, 0.04% Bromophenol blue, and 10% glycerol). Samples were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at 110 V for 1.5 h and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was soaked in PBS with 5% nonfat dry milk for 2 h at 4 °C to saturate irrelevant protein binding sites and then incubated with specific antibodies against CYP2E1 (GeneTex, Irvine, CA, USA) and NFκB P65, iNOS, COX-2, JNK, P38, ERK1/2, and GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After treatment with the primary antibodies in membranes were washed four times by PBS-Tween solution for 1 h and then incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). Protein bands were visualized with a chemiluminescence (ECL) kit (Millipore Corp., Billerica, MA, USA) and analyzed by using an LAS-4000 mini (Fujifilm Co., Tokyo, Japan).

Histopathological Analysis. Fresh liver tissues were fixed in 10% neutral-buffered formalin solution, dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin. These paraffin sections were later sectioned using a microtome and stained with hematoxyl and eosin (H&E) solution. Photomicrographs were imaged by using ImageJ software version 1.45h (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Histological activity index (HAI) scoring was applied to evaluate liver damage.[24]

Statistical Analysis. The values are expressed as the mean ± SEM. A significant difference was used at the 0.05 probability level. One-way analysis of variance and the least significant difference test were used to differentiate the differences between treatments. All statistical analyses of data were performed using SAS (SAS Institute Inc., Cary, NC, USA; 2002).

RESULTS

Effects of NJ on Growth Performance, Serum Biochemical Values, Liver Lipids, and Daily Fecal Lipid/Bile Acid Outputs. After 1 year of fermentation, the contents of phenols, flavonoids, and condensed tannins in collected NJ were 79.57 mg GAE/100 mL, 12.71 mg CE/100 mL, and 16.67 mg CE/100 mL, respectively, whereas there were 23.36 and 2141.52 mg/100 mL ascorbic acid and crude polysaccharide in the NJ, respectively (Table 1). With regard to the mineral profile in NJ, the major component was potassium (K), followed by magnesium (Mg), and sodium (Na). Interestingly, zinc (Zn), manganese (Mn), and selenium (Se) were also analyzed in NJ. After 4 weeks of experiment, lower ($p < 0.05$) final body weights of mice were observed in the alcohol-diet groups (alcohol, Alc+NJ_1X, Alc+NJ_2X, and Alc+NJ_3X) as compared to the control group (Table 2). Among the differences in relative sizes of heart, liver, kidney, and visceral fat, only a smaller ($p < 0.05$) size of visceral fat was measured in alcohol-diet groups than in the control group, but the opposite results were observed in sizes of liver and kidney. Although alcohol-diet fed groups had higher ($p < 0.05$) serum triacylglycerol levels, an alcohol liquid diet did not ($p > 0.05$) affect serum cholesterol levels compared to the control group. In liver lipid contents, NJ supplementation decreased ($p < 0.05$) the triacylglycerol and cholesterol contents in alcohol-diet groups, whereas cholesterol levels in alcohol-diet fed mice with

Table 1. Phytochemical and Mineral Contents in Noni Juice

	content[a]
total phenols (mg GAE[b]/100 mL)	79.57 ± 2.89
total flavonoids (mg CE[b]/100 mL)	12.71 ± 1.82
condensed tannins (mg CE/100 mL)	16.67 ± 0.72
ascorbic acid (mg/100 mL)	23.36 ± 0.60
crude polysaccharides (mg/100 mL)	2141.52 ± 68.98
minerals	
potassium (K) (mg/100 mL)	376.02 ± 9.96
magnesium (Mg) (mg/100 mL)	25.31 ± 0.64
sodium (Na) (mg/100 mL)	22.31 ± 0.49
calcium (Ca) (mg/100 mL)	2.89 ± 0.08
iron (Fe) (mg/100 mL)	0.33 ± 0.01
zinc (Zn) (mg/100 mL)	0.16 ± 0.02
manganese (Mn) (mg/100 mL)	0.12 ± 0.01
selenium (Se) (μg/100 mL)	0.07 ± 0.00

[a]The data are given as the mean ± SEM ($n = 3$). [b]GAE, gallic acid equivalent; CE, catechin equivalent.

dx.doi.org/10.1021/jf4038419 | J. Agric. Food Chem. 2013, 61, 11016−11024

Table 2. Growth Performance, Relative Sizes of Organs and Visceral Fat, Serum Biochemical Values, Liver Lipids, and Daily Fecal Lipid/Bile Acid Outputs of Experimental Mice[a]

	group				
	Control	Alcohol	Alc+NJ_1X	Alc+NJ_2X	Alc+NJ_3X
Growth Performance					
initial body weight (g)	22.38 ± 0.41a	22.01 ± 0.27a	21.65 ± 0.45a	21.74 ± 0.46a	22.69 ± 0.39a
final body weight (g)	28.22 ± 0.58a	24.92 ± 0.34b	24.38 ± 0.45b	24.18 ± 0.62b	25.56 ± 0.43b
Relative Size					
heart (g/100 g body weight)	0.52 ± 0.02a	0.53 ± 0.02a	0.49 ± 0.03a	0.51 ± 0.02a	0.49 ± 0.02a
liver (g/100 g body weight)	3.44 ± 0.04b	4.16 ± 0.11a	4.21 ± 0.13a	4.16 ± 0.10a	4.17 ± 0.07a
kidney (g/100 g body weight)	0.99 ± 0.04b	1.22 ± 0.04a	1.25 ± 0.04a	1.18 ± 0.04a	1.24 ± 0.02a
visceral fat (g/100 g body weight)	3.35 ± 0.22a	2.01 ± 0.15b	1.90 ± 0.09b	2.01 ± 0.09b	1.92 ± 0.13b
Serum Biochemical Values					
triacylglycerol (mg/dL)	47.85 ± 2.09b	58.31 ± 2.40a	54.08 ± 2.56ab	55.13 ± 1.90a	56.45 ± 2.43a
cholesterol (mg/dL)	118.44 ± 3.07a	118.33 ± 1.83a	114.77 ± 2.10a	115.41 ± 2.77a	114.80 ± 3.45a
AST (U/L)	49.00 ± 2.40c	75.42 ± 2.72a	49.25 ± 2.18c	62.83 ± 1.95b	50.25 ± 2.64c
ALT (U/L)	13.92 ± 0.48c	20.50 ± 0.97a	17.50 ± 0.98b	18.00 ± 0.58b	16.33 ± 0.73b
alcohol (mg/dL)	3.92 ± 0.19c	10.00 ± 0.28a	5.08 ± 0.26b	4.67 ± 0.22b	4.58 ± 0.23bc
Liver Lipids					
triacylglycerol (mg/g liver)	13.49 ± 0.69c	19.59 ± 0.91a	15.14 ± 0.78bc	16.90 ± 0.72b	16.67 ± 1.04b
cholesterol (mg/g liver)	1.76 ± 0.09b	2.51 ± 0.14a	1.64 ± 0.08b	1.80 ± 0.12b	1.91 ± 0.09b
Daily Fecal Lipid/Bile Acid Outputs					
triacylglycerol (mg/mouse/day)	2.24 ± 0.23c	1.98 ± 0.28c	4.64 ± 0.16a	4.08 ± 0.37ab	3.40 ± 0.25b
cholesterol (mg/mouse/day)	2.13 ± 0.26b	1.93 ± 0.30b	3.69 ± 0.42a	3.66 ± 0.77a	4.10 ± 0.09a
bile acid (μmol/mouse/day)	1.56 ± 0.19b	1.44 ± 0.39b	1.98 ± 0.22ab	2.61 ± 0.20a	2.56 ± 0.28a

[a]The data are given as the mean ± SEM (n = 12; feces, n = 3). Mean values with different letters are significantly different ($p < 0.05$).

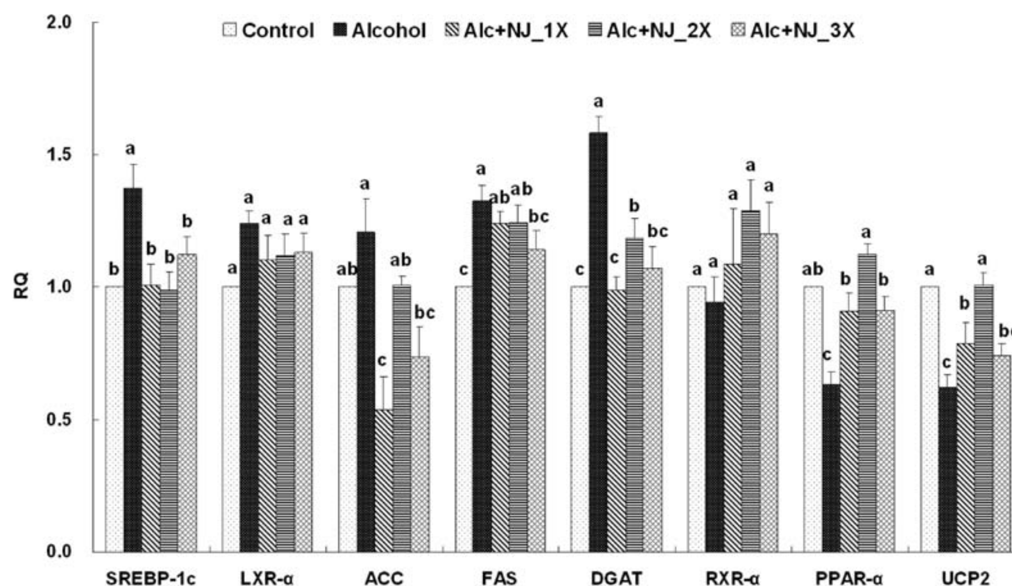

Figure 1. mRNA expressions of SREBP-1c, LXR-α, ACC, FAS, DGAT, RXR-α, PPAR-α, and UCP2 in livers of experimental mice. Data are given as the mean ± SEM (n = 12). Bars with unlike letters in each target gene differ significantly in each gene ($p < 0.05$).

NJ were even similar ($p > 0.05$) than those of the control group. Moreover, daily fecal triacylglycerol, cholesterol, and bile acid outputs of alcohol-diet fed mice were increased ($p < 0.05$) by supplementing NJ.

Effects of NJ on de Novo Lipogenesis of Livers. With regard to the de novo lipogenesis (Figure 1), increased ($p < 0.05$) gene expressions, that is, SREBP-1c, ACC, FAS, and DGAT, were observed in alcohol groups relative to those in the control group.

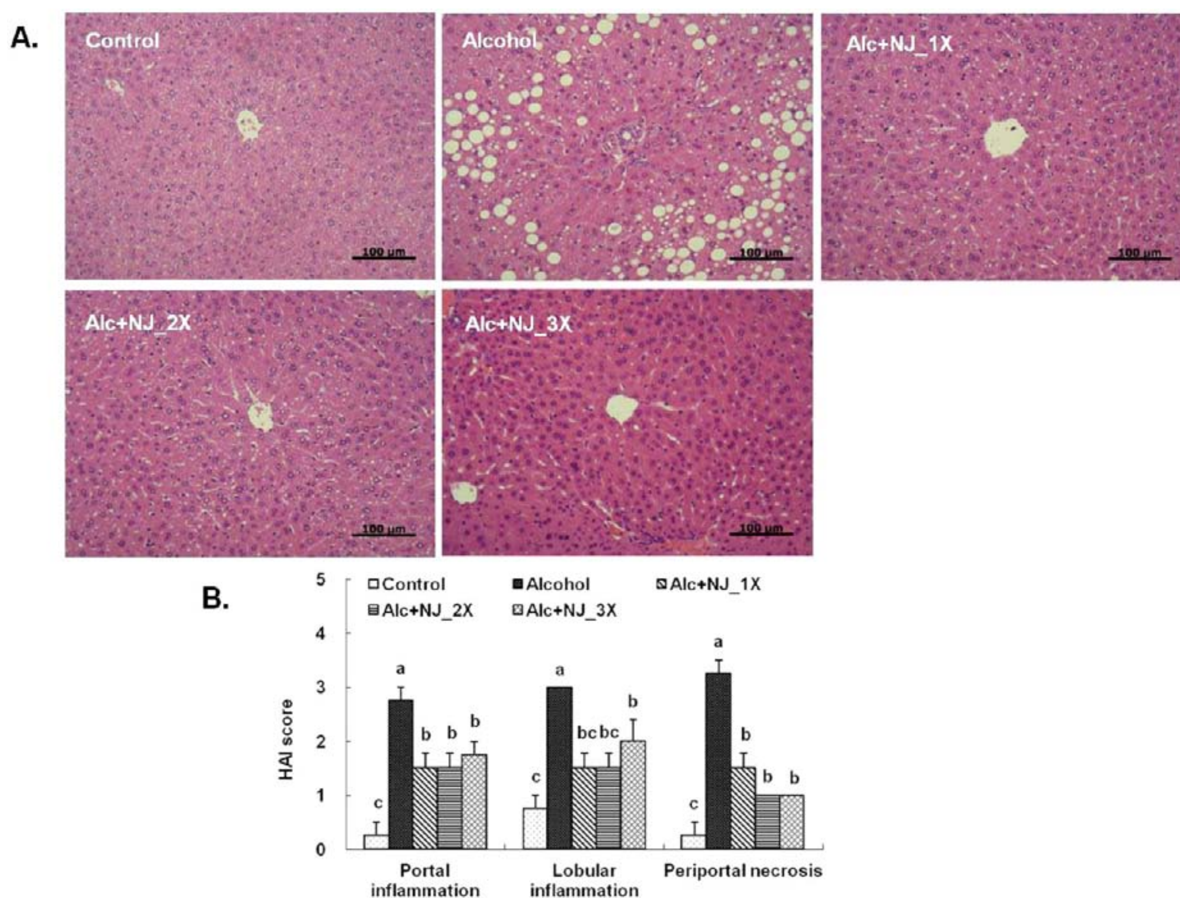

Figure 2. H&E stain of liver tissues: (A) gross appearance of representative livers in experimental mice (scale shown in 100 μm); (B) HAI scores of liver portal, lobular, and portal peripheral inflammation (data are given as the mean ± SEM (n = 12); bars with unlike letters in each score differ significantly in each score ($p < 0.05$)).

Table 3. Liver Antioxidant Capacities and Cytokine Excretion of Experimental Mice[a]

	Control	Alcohol	Alc+NJ_1X	Alc+NJ_2X	Alc+NJ_3X
			Antioxidant Capacities		
TBARS (nmol MDA equiv/mg protein)	0.38 ± 0.02c	0.75 ± 0.02a	0.44 ± 0.02b	0.45 ± 0.02b	0.48 ± 0.01b
GSH (nmol/mg protein)	71.31 ± 3.33b	59.41 ± 2.26c	80.35 ± 2.12a	86.55 ± 1.97a	72.62 ± 1.86b
TEAC (nmol/mg protein)	70.94 ± 1.35b	61.80 ± 1.98c	78.69 ± 1.87a	75.30 ± 2.28ab	76.25 ± 2.52ab
SOD (munits/mg protein)	53.25 ± 2.55a	52.55 ± 2.22a	53.82 ± 2.91a	57.97 ± 3.20a	50.49 ± 2.04a
GSH-Px (nmol NADPH oxidized/min/mg protein)	70.73 ± 2.92b	81.39 ± 4.10ab	91.36 ± 4.37a	91.38 ± 4.95a	91.90 ± 7.62a
CAT (units/mg protein)	183.51 ± 8.92a	185.96 ± 5.66a	198.95 ± 9.02a	200.37 ± 5.31a	198.53 ± 10.74a
			Cytokine		
TNF-α (pg/mg protein)	15.88 ± 0.46c	25.47 ± 0.75a	20.71 ± 1.14b	21.49 ± 1.00b	21.74 ± 1.02b
IL-1β (pg/mg protein)	4.16 ± 0.12c	5.03 ± 0.12a	4.37 ± 0.15bc	4.51 ± 0.17bc	4.71 ± 0.17ab

[a]Data are given as the mean ± SEM (n = 12). Mean values with different letters are significantly different ($p < 0.05$).

However, NJ supplementation significantly down-regulated ($p < 0.05$) SREPB-1c, ACC, and DGAT expressions compared to those of the alcohol group, whereas there were tendencies toward lower expressions of LXR-α and FAS in alcohol-diet fed mice with NJ. With regard to the β-oxidation of fatty acids, although RXR-α expression was not ($p > 0.05$) affected among groups, PPAR-α and UCP2 expressions in alcohol-diet fed mice were up-regulated ($p < 0.05$) by supplementing NJ. Meanwhile, the larger lipid droplets were observed in the liver section of the alcohol group compared to the control group, but NJ supplemented groups apparently had smaller lipid droplets or even no lipid droplets (Figure 2A), which also confirms the hepatic lipid contents in Table 1.

Effects of NJ on Lipid Peroxidation and Antioxidant Capacities in Livers. Chronic alcohol consumption (alcohol-diet groups) significantly increased ($p < 0.05$) liver TBARS values, but NJ supplementation decreased ($p < 0.05$) them (Table 3). In the antioxidant status, lower ($p < 0.05$) TEAC and GSH levels were demonstrated in the alcohol group compared to those in the control group. However, NJ supplementation enhanced ($p < 0.05$) those two values to similar to those of the control group. Although NJ supplementation seems not ($p > 0.05$) to be effective on enzymatic antioxidant systems (SOD, GSH-Px, and CAT), there were tendencies toward higher GSH-Px and CAT activities in alcohol-diet fed mice supplemented with NJ.

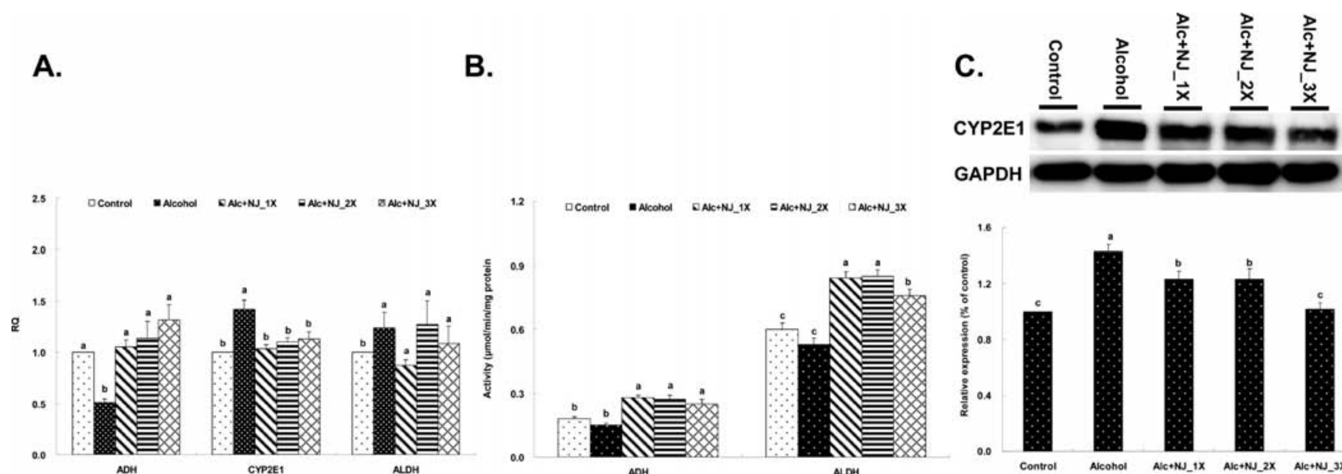

Figure 3. Liver (A) ADH, ALDH, and CYP2E1 mRNA levels, (B) ADH and ALDH activities, and (C) CYP2E1 protein level of experimental mice. Data are given as the mean ± SEM ($n = 12$). Bars with unlike letters in each target enzyme differ significantly in each gene ($p < 0.05$).

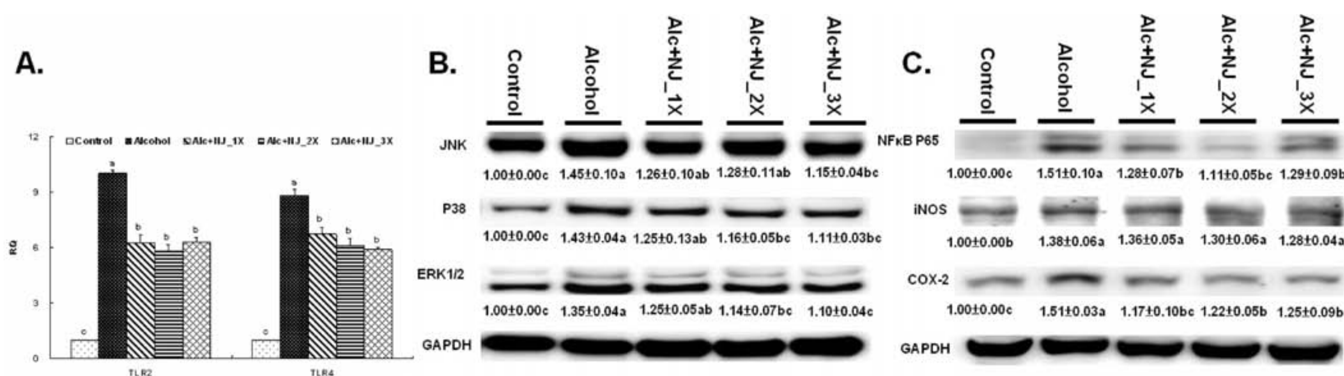

Figure 4. Liver (A) TLR2 and TLR4 mRNA levels, (B) JNK, P38, and ERK 1/2 protein levels, and (C) NFκB P65, iNOS, and COX-2 protein levels of experimental mice. Data are given as the mean ± SEM ($n = 12$). Bars or mean values with unlike letters in each target gene or protein differ significantly in each gene ($p < 0.05$).

Effects of NJ on Alcohol Metabolism in Livers. To understand the effects of NJ on alcohol mechanism in livers, serum alcohol level (Table 1) and gene expressions and activities or protein expression of ADH, ALDH, and CYP2E1 were investigated (Figure 3). Chronic alcohol consumption resulted in higher serum alcohol levels, but NJ supplementation reduced ($p < 0.05$) them. The alcohol group had lower ($p < 0.05$) ADH expression and higher ($p < 0.05$) CYP2E1 expression but no ($p > 0.05$) effect on ALDH expression (Figure 3A) compared to the control group. Meanwhile, NJ supplementation up-regulated ($p < 0.05$) ADH gene expressions but down-regulated ($p < 0.05$) CYP2E1 expressions in alcohol-diet fed mice. In the activities (Figure 3B) and protein expression (Figure 3C), NJ supplementation increased ($p < 0.05$) ADH and ALDH activities but decreased ($p < 0.05$) CYP2E1 protein levels in alcohol-diet fed mice.

Effects of NJ on Liver Damage and Inflammatory Responses. Serum AST and ALT values were increased ($p < 0.05$) by the alcohol liquid diet but decreased ($p < 0.05$) by supplementing NJ (Table 2). Similar patterns of liver TNF-α and IL-1β protein levels are demonstrated in Table 3. On the basis of the histopathological analysis of livers, higher ($p < 0.05$) HAI scores in portal, lobular, and portal peripheral inflammations were obtained in alcohol-diet fed groups compared to those of the control group (Figure 2B). However, those HAI scores were alleviated ($p < 0.05$) by NJ supplementation. With regard to the mechanism of inflammatory responses in an alcohol diet (Figure 4), chronic alcohol consumption up-regulated ($p < 0.05$) the gene expressions of TLR2 and TLR4, as well as protein levels of JNK, P38, ERK1/2, NFκB P65, iNOS, and COX-2 compared to those of the control group. Figure 4A illustrates that NJ supplementation down-regulated ($p < 0.05$) TLR2 and TLR4 gene expressions. Meanwhile, NJ supplementation also reduced ($p < 0.05$) protein levels of JNK, P38, and ERK 1/2 in alcohol-diet mice, where the higher dosage of NJ showed more significant impacts on them (Figure 4B). In addition, NJ supplementation lowered ($p < 0.05$) NFκB P65 and COX-2 protein levels in alcohol-diet fed mice (Figure 3C).

■ DISCUSSION

AFLD was successfully developed by Lieber-DeCarli alcohol liquid diet on rodents and, meanwhile, activated NADPH oxidase, the initiation of hepatic fat infiltration, and increased hepatic levels of toxic lipid peroxidation products such as 4-hydroxynonenal (4-HNE) and MDA were observed.[2] There are two reasons to explain the lower body weight induced by chronic alcohol consumption: (1) morphological changes in hepatic mitochondria, thus reducing ATP syntheses;[25] (2) oxidation of alcohol via microsomal ethanol-oxidizing system, thus depleting NADPH.[26] Previous studies indicated that NJ contains many bioactive compounds including polysaccharides, flavonoids, phytosterols, vitamin C, carotenoids, etc.[11−13] Moreover,

previous research attributed the hepatoprotection of polysaccharides extracted from *Lycium barbarum* against alcohol in rats to decreased serum lipid levels, oxidative stress, and liver damage.[27] According to our previous studies,[16,17] an amelioration of NJ on serum lipids and oxidative status in high-cholesterol/fat dietary hamsters is related to the phytochemicals in NJ. Besides, daily fecal lipid/bile acid outputs represent the true triacylglycerol and cholesterol excretions. Therefore, it is supposed that decreased liver lipids in alcohol-diet fed mice with NJ partially result from increased daily fecal lipid and bile acid outputs. On the basis of our knowledge, macrominerals, that is, K, Mg, Na, and calcium (Ca), are necessary and beneficial to human health, whereas trace minerals, Zn, Mn, and Se, are important as well. For example, Mn and Se are cofactors for SOD and GSH-Px, respectively. Maintenance of zinc concentration in serum and liver can decrease liver fibrosis.[28] Hence, hepatoprotection of NJ against alcohol consumption may be highly related to its bioactive components.

Polyphenols from the plant kingdom demonstrated hypolipidemic and obesity effects through decreasing lipogenesis (up-regulations of SREBP-1c, ACC, FAS, etc.) or increasing energy expenditure (up-regulations of RXR-α, PPAR-α, UCP2, etc.).[4,6] Our previous studies also identified phenolic acids as the major polyphenolic compounds, not flavonoids, by HPLC analysis.[16,17] Moreover, NJ down-regulates SREBP-1c but up-regulates PPAR-α and UCP2, which results in decreased serum lipids in high-fat/cholesterol diet hamsters. Abnormal de novo lipogenesis and suppressed mitochondrial β-oxidation in livers were reported in chronic alcohol treatment, which contribute to hepatosteatosis development.[2] On the basis of current data, we conjecture that lipid contents and droplets in livers of alcohol-diet fed mice supplemented with NJ are highly related to down-regulation of de novo lipogenesis and up-regulation of β-oxidation by NJ supplementation (Table 2; Figures 1 and 2A).

During alcohol metabolism by CYP2E1, ROS also generate and increase oxidative stress in the liver, which accelerates hepatocyte damage. GSH is the major endogenous antioxidant in hepatocytes, whereas SOD, CAT, and GSH-Px are in charge of counteracting ROS and hydrogen peroxide (H_2O_2) in biological systems. It was reported that dietary Se can increase GSH-Px activities.[29] Recently, our study also indicated that enhanced liver antioxidant capacities in high-cholesterol/fat dietary hamsters supplemented with NJ result from the polyphenol contents in NJ.[17] Therefore, the bioactive compounds (polyphenol, polysaccharide, and Se) in NJ may contribute increased liver antioxidant capacities in alcohol-diet fed mice (Tables 1 and 3).

In the liver, alcohol is mainly metabolized by cytosolic ADH to acetaldehyde, which is further metabolized by mitochondrial ALDH to acetate. Regardless of ADH in an alcohol metabolism system, there are another two enzymatic systems to metabolize alcohol: (1) CAT in the peroxisomes and (2) microsomal ethanol oxidizing system (MEOS) in microsomes. The major component of MEOS is CYP2E1, which is thought to be one of the main sources in alcohol-induced ROS.[3] Aged black garlic contains many polyphenols, and its hepatoprotection against chronic alcohol consumption is attributed to decreased CYP2E1 activity and increased antioxidant capacity.[30] In addition, polysaccharides were identified as one of the major bioactive components in *Antrodia camphorata*. It was reported that *A. camphorata* can increase hepatic ALDH and CAT activities, which accelerate alcohol clearance and lessen liver damage in continuously alcohol fed rats.[21] Moreover, the hepatic TEAC and GSH levels in alcohol-fed mice were also enhanced by supplementing NJ in the current study. Therefore, it is conjectured that the lower serum alcohol levels in alcohol-diet fed mice supplemented with NJ result from higher ADH and ALDH expressions or activities; meanwhile, lower TBARS values should be also associated with the down-regulation of CYP2E1 protein levels (Table 3 and Figure 3C).

An anti-inflammatory effect of noni was discussed by Yu et al.[14] They identified quercetin, scopoletin, β-sitosterol, campesterol, and ursolic acid as the five major compounds isolated from the ethyl acetate fraction in noni paste and indicated that quercetin and scopoletin show significant reductions in PGE_2, IL1β, and IL-6 levels in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation. However, there are several pathways contributing to alcohol-induced inflammatory responses, for example, an activation of MAPKs, which increases cytokine formation. Acetaldehyde, an intermediary metabolite during alcohol metabolism, can increase NFκB P65 and then combine with the α_2 (I) collagen promoter, further enhancing NFκB by a mechanism dependent on the accumulation of H_2O_2.[31] The wild-type mice fed Leiber-DeCarli alcohol liquid diets showed increased hepatic TLR2 and TLR4 gene expressions, which are related to liver inflammation and injury.[32] In addition, previous studies summarized that either AFLD patients or animal models had higher gut-derived endotoxin levels, which induces NFκB activation and secretes pro-inflammatory cytokines (TNF-α) via a TLR4-mediated regulation.[33] *Agrimonia eupatoria* against chronic ethanol-induced liver injury in rats was due to a suppression of TLR4-mediated inflammatory signaling including transcription factor NFκB activation and NFκB associated with inflammatory mediators (TNF-α, iNOS, and COX-2).[10] In lipopolysaccharide-stimulated macrophages, *Astragalus* polysaccharides down-regulated the phosphorylation of ERK and JNK and then suppressed NFκB activation, which implicates decreased secretions of TNF-α and IL-1β.[34] Zinc supplementation had a favorable effect on fibrosis in vitro and in vivo, where the protective mechanism is through acting as an antioxidant and down-regulating MAPK, transforming growth factor beta (TGF-β), and NFkappaB/ikappaB-alpha complex signaling pathway in alcohol-induced activation of hepatic stellate cells.[27] Therefore, suppressive effects of NJ on TLR-mediated inflammatory responses including MAPK pathways, and NFκB and COX-2 in alcohol-diet fed mice could be attributed to its hepatoprotection.

Chronic alcohol consumption induced liver abnormality (steatosis) and its associated pathogenesis (inflammation). NJ naturally fermented for 1 year contains many polysaccharides, polyphenols, and some trace minerals (Mn and Se). Via feeding mice with an alcoholic liquid diet for 4 weeks, hepatoprotection of NJ against chronic alcohol consumption was attributed to the following: (1) decreased liver lipid accumulation due to down-regulated de novo lipogenesis (SREBP-1c, ACC, and DAGT), up-regulated β-oxidation of fatty acids (PPAR-α and UCP2), and increased daily fecal lipid/bile acid outputs; (2) increased antioxidant capacity due to enhanced antioxidative defense system (GSH and TEAC levels) and decreased CYP2E1 activity; (3) accelerated alcohol clearance due to up-regulated ADH and ALDH expressions or activities; and (4) alleviated inflammation due to regulated TLR-mediated inflammatory responses (Figure 5). Besides, our results demonstrated that there is not a dose-dependent beneficial effect of noni juice against chronic alcohol consumption. This phenomenon may be due to the bioavailability of putative

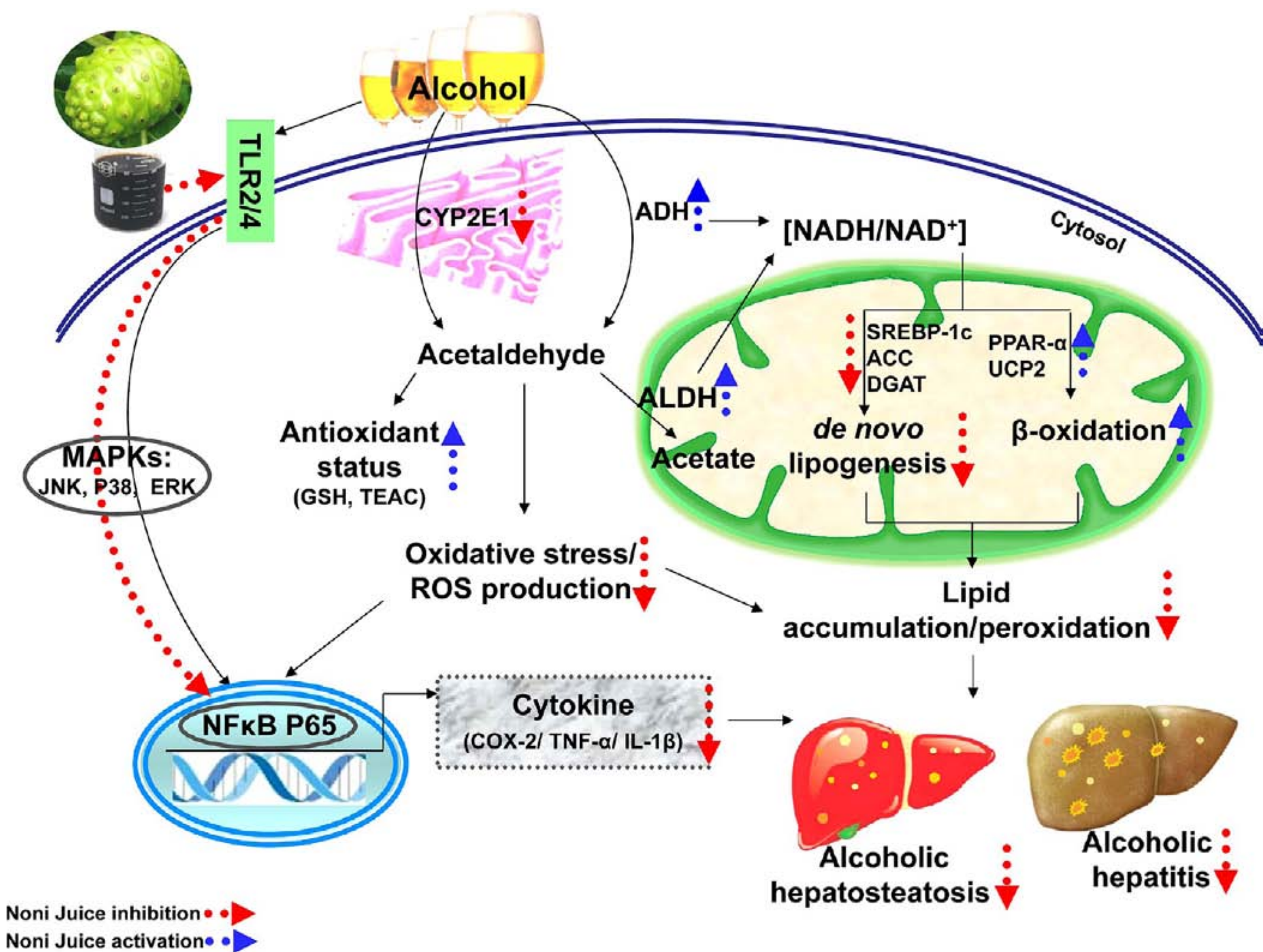

Figure 5. Schematic representation of action mechanism by which noni juice alleviates chronic alcohol-induced liver damage. Four protective effects are involved: (1) NJ down-regulates de novo lipogenesis (SREBP-1c, ACC, and DAGT), up-regulates β-oxidation of fatty acids (PPAR-α and UCP2), and increases daily fecal lipid/bile acid outputs; (2) NJ increases antioxidative status (GSH, TEAC levels) and decreases ROS generation by CYP2E1 activity; (3) NJ accelerates alcohol clearance via up-regulating hepatic ADH and ALDH; (4) NJ reduces inflammation via regulation of TLRs-mediated inflammatory responses including MAPKs pathways and NFκB P65, COX-2, TNF-α, and IL-1β productions. SREBP-1c, sterol regulatory element binding protein-1c; ACC, acetyl CoA carboxylase; DAGT, diglyceride acyltransferase; PPAR-α, proliferator-activated receptor-alpha; UCP2, uncoupling protein 2; GSH, glutathione; TEAC, trolox equivalent antioxidant capacity; ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; TLRs, Toll-like receptors; CYP2E1, cytochrome P4502E1; MAPK, mitogen-activated protein kinases; NFκB P65, nuclear factor kappa B P65; COX-2, cyclooxygenease-2; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta.

ingredients in noni juice. Hence, a dose of 5 mL NJ (including 107.08 mg of crude polysaccharides)/kg BW is recommended to protect the liver against chronic alcohol consumption.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ACC, acetyl CoA carboxylase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalse; COX-2, cyclooxygenease-2; CYP2E1, cytochrome P450, subfamily e, polypepetide 1; DAGT, diglyceride acyltransferase; ERK1/2, extracellular signal-regulated kinase 1/2; GSH, glutathione; GSH-Px, glutathione peroxidase; IL-1β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; NFκB P65, nuclear factor kappa B P65; NJ, noni juice; PPAR-α, proliferator-activated receptor-alpha; SREBP-1c, sterol regulatory element binding protein-1c; TEAC, trolox equivalent antioxidant capacity; TBARS, triobarbituric acid reactive substances; TLRs, Toll-like receptors; TNF-α, tumor necrosis factor-alpha; UCP2, uncoupling protein 2

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