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Original Article

Hepatoprotective effects of naturally fermented noni juice against thioacetamide-induced liver fibrosis in rats

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Abstract

Background: Excessive reactive oxygen species (ROS) can result in inflammation and cytokine secretion in the liver, and then activate hepatic stellate cells that cause the accumulation of extracellular matrix proteins, especially collagen, in liver tissue. Naturally fermented noni juice (NJ; *Morinda citrifolia*) has been used for decades as a nutraceutical in humans. In this study, we intended to examine if NJ can ameliorate ROS-induced liver fibrosis via a thioacetamide (TAA)-induced rat model.

Methods: The 50 rats used in this study were separated into five groups of 10 rats each for 8 weeks as follows: (1) control group; (2) TAA; (3) TAA + low-dose NJ (2.51 mL NJ/kg); (4) TAA + medium-dose NJ (5.02 mL NJ/kg); and (5) TAA + high-dose NJ (7.52 mL NJ/kg).

Results: Treatment with TAA resulted in lower body weight and serum lipid levels ($p < 0.05$), while liver weight and collagen contents, and serum alanine aminotransferase and aspartate aminotransferase values were increased ($p < 0.05$). The protective effects of NJ on TAA treatment resulted from decreased endoplasmic reticulum stress-related gene expressions ($p < 0.05$), inflammatory cytokines, collagen accumulation, and matrix metalloproteinase (MMP-2 and MMP-9) activities, as well as upregulated ($p < 0.05$) tissue inhibitors of metalloproteinase (TIMP-1 and TIMP-3) in livers. NJ also increased hepatic antioxidant capacities ($p < 0.05$).

Conclusion: Naturally fermented NJ manifests a protective potential on liver fibrosis via the enhancement of antioxidant capacities, as well as decreasing endoplasmic-reticulum stress and MMP-2/MMP-9 activities.

Keywords: antioxidant capacity; ER stress; liver fibrosis; MMP-2/MMP-9 activity; noni juice

1. Introduction

Most chronic liver injuries including alcoholic disorder, viral hepatitis, biliary obstruction, or hemochromatosis consequently lead to hepatic fibrosis, a critical step which is instrumental in deciding the clinical outcome of liver disease.[1] The liver can function to facilitate the biochemical conversion of administered substances which significantly increase reactive oxygen species (ROS) generation.[2] A single dose of

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thioacetamide (TAA), a hepatotoxic agent, could produce centrilobular hepatic necrosis, while a chronic administration can lead to fibrosis or cirrhosis.[3] It is assumed that oxidative stress contributes to the development of TAA-induced liver fibrosis.[4] It has also been suggested that ROS is one of the important factors in cytokine-induced liver fibrogenesis by TAA induction.[5] A high ROS level effectively induces apoptosis, probably through an activation of the endoplasmic-reticulum (ER) stress-induced apoptotic pathway.[6] While transient and low-grade ER stress can be overcome by the unfolded protein response, persistent and severe ER stress results in cell apoptosis and also stimulates inflammatory responses.[7] Antioxidant supplements may emerge as potentially antifibrotic agents by either protecting hepatocytes from ROS or inhibiting the activation of hepatic stellate cells (HSCs).[8] Our previous reports indicated that enhanced liver antioxidant capacities in high-cholesterol/fat dietary hamsters[9] or alcohol-diet fed mice[10] supplemented with noni juice (*Morinda citrifolia*) (NJ) result from the polyphenolic contents in NJ. In addition, an excessive accumulation of extracellular matrix proteins (collagen) is often observed in liver fibrosis.[11] The injured liver cells stimulate HSCs to transform into myofibroblast-like cells which secrete large amounts of collagen, thereby producing liver fibrosis. Increasingly, ROS are viewed as a candidate driver of HSC activation and collagen I upregulation.[12] However, downstream mediators for the ROS on the activation of HSCs and the increased collagen synthesis could be a potential avenue to alleviate liver fibrosis and inflammation.

Polysaccharides, fatty acid esters, glycosides, iridoids, anthraquinones, flavonoids, phytosterols, carotenoids, vitamin A, antraquinones, potassium, and others have been identified as putative active ingredients in NJ.[13] Our previous report indicated that gentisic, *p*-hydroxybenoic, and chlorogenic acids have been characterized as the major phenolic acids in our fermented NJ, while the hepatic antioxidant and antiinflammation effects of NJ in a high-fat diet were partially attributed to its phenolic acid.[9] Furthermore, the major mineral in NJ is potassium (K), followed by magnesium (Mg), and sodium (Na). Interestingly, some trace minerals, i.e., zinc (Zn), manganese (Mn), and selenium (Se) were also found in this fermented NJ.[10] In addition, this naturally fermented NJ contains polysaccharides (2141.52 mg/100 mL), and its anti-inflammatory effects against alcoholic liver disease also significantly result from its polysaccharide contents. It has been reported that polysaccharides can downregulate the phosphorylation of ERK and JNK, and then suppress NFκB activation, which influences tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) secretions.[14] Therefore, we speculated that the bioactive compounds (polyphenols, polysaccharides, and minerals) in the naturally fermented NJ may also contribute to increased hepatic antioxidant capacities and antiinflammatory responses in TAA-induced liver fibrosis.

Although NJ showed hypolipidemic, antioxidative, and antiinflammatory effects in a high-fat/cholesterol diet[9] and liquid alcohol diet[10] fed to hamsters and mice, respectively, the protective mechanism of NJ against TAA-induced rat liver fibrosis is still lacking. Therefore, by employing a TAA-induced liver fibrosis rat model, the present study addressed the protective effects of NJ via: (1) increased antioxidative capacities; (2) downregulation of inflammatory and ER stress; and (3) inhibited collagen accumulation.

2. Methods

2.1. NJ preparation

NJ was sourced from the same batch as the one used in our previous studies.[9,10] On the basis of our previous studies,[10] the major identified phenolic acids in NJ are gallic acid, gentisic acid, chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, ferulic acid, and *p*-anisic acid. Identified flavonoids in NJ include epicatechin, hesperidin, and naringin. Gentisic, *p*-hydroxybenzoic, and chlorogenic acid were the dominant phenolic acids in NJ. To ensure accurate measurements of phenolic acid, flavonoids, condensed tannin, ascorbic acid, and polysaccharides were obtained, the NJ stored at −20°C for 1 year was measured based on the previous methods.[10]

2.2. Animals and experimental design

Fifty male Wistar rats (6 weeks old, 200–220 g) were purchased from BioLASCO Taiwan Co. Ltd. (Taipei, Taiwan), and acclimated under an environmentally controlled room at 22 ± 2°C and 12/12-h light/dark cycle. After 1 week of acclimation, the 50 rats were randomly divided into five groups: (1) the control group: intraperitoneal (i.p.) saline + normal distilled water (NDW) (ddH_2O); (2) TAA (i.p.); (3) TAA (i.p.) + low-dose NJ [NJ-L; rats were given 2.51 mL NJ/kg body weight (BW) orally]; (4) TAA (i.p.) + medium-dose NJ (NJ-M; rats were given 5.02 mL NJ/kg BW orally); and (5) TAA (i.p.) + high-dose NJ (NJ-H; rats were given 7.52 mL NJ/kg BW orally). The doses and schedules of NJ were calculated, compared, and associated with the dose from our previous report[10] involving mice and rats.[15] During the experimental period, liver fibrosis was induced in rats by i.p. administration of TAA (100 mg/kg) three times weekly on Monday, Wednesday, and Friday; and the ddH_2O or NJ oral gavages on Tuesday, Thursday, and Saturday. TAA was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in sterile saline. Ultimately, body weight, liver weight, serum biochemical values, and histopathological results were analyzed at the end of the 8-week experiment.

Rats were fasted overnight (approximately 10 hours) and then sacrificed by CO_2 asphyxiation on the last experimental day. Blood was collected for biochemical analyses and other measurements, and livers were removed and individually weighed. The liver tissues were fixed or stored later in Bouin's solution or RNA (Ambion, Austin, TX, USA) in a deep freezer (−70°C) for further analyses. For this study, the animal use and protocol were reviewed and approved by the National Taiwan University Animal Care and Use Committee (IACUC No. 100-101).

2.3. Determination of serum biochemical values and hepatic lipids

The serum biochemical values, that is, triacylglycerol (TAG), total cholesterol (TC), 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). Hepatic TAG was measured using commercial kits (Randox Laboratories Ltd., Antrim, UK) according to the previous method.[11]

2.4. Collagen content in livers

The collagen assay was performed according to the procedure as described by a previous method with a slight modification.[16] The concentration of hydroxyproline (Sigma-Aldrich) is representative of a value for collagen because every 7.46 g of collagen contains 1 g of hydroxyproline. Prior to analysis, three essential buffers were required, and the details are described below:

1. Acetate–citrate buffer (pH = 6.5) which contains 120 g sodium acetate trihydrate, 46 g citric acid, 12 mL acetic acid, and 34 g sodium hydroxide in 1 L deionized water.
2. Chloramine-T (0.056M) which contains 1.27 g chloramine-T, 20 mL 50% N-propanol in 1 L deionized water.
3. Ehrlich's reagent, which requires 15 g dimethyl amino-benzaldehyde in 100 mL solvent consisting of N-propanol and perchloric acid (2:1 in volume).

All chemicals in this analysis were purchased from Sigma-Aldrich, Inc. Briefly, the serial dilution of hydroxyproline stock was added into a 96-well plate as standard, and the liver homogenates were also added. They were then autoclaved at 120°C for 20 minutes. Finally, the hydroxyproline in liver homogenates was oxidized into pyrrole by chloramine-T, and tested with Erlich's reagent for color reaction. The liver collagen level was calculated by hydroxyproline × 7.46.

2.5. Preparation of liver homogenate

The liver homogenate (10%, w/v) was made with phosphate buffered saline (pH 7.0, containing 0.25M sucrose), and the supernatant was collected by centrifugation at 2,4148 g for 30 minutes. The protein content in the supernatant was measured according to the procedures of a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Determination of liver lipid peroxidation level and antioxidant capacity

The liver thiobarbituric acid reactive substances (TBARS) level was used to determine liver lipid peroxidation, whereas glutathione (GSH), and activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were assayed as indices for liver antioxidant capacities. The assayed methods were conducted according to the earlier referenced methods.[10] The liver TBARS level, reduced GSH content, as well as activities of SOD, CAT, and GSH-Px were expressed as nmole malondialdehyde equivalent/mg protein, nmole/mg protein, munit/mg protein, unit/mg protein, and nmole NADPH oxidized/min/mg protein, respectively.

2.7. Determination of hepatic TNF-α and IL-1β levels

Hepatic 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 milligram of protein by using standard curves.

2.8. Histopathological Analysis

The liver tissues were fixed in neutral-buffered formalin solution for no more than 24 hours, dehydrated in graded alcohol, cleared in xylene and then embedded in paraffin. Histopathological examination was performed by hematoxylin and eosin (H&E) and Masson's trichrome staining. The liver damage was measured by a double-blind test according to routine histological activity index scoring.[17]

2.9. Real-time polymerase chain reaction

Total RNA was isolated from the stored frozen liver tissues by using the protocol described using Rneasy Mini Kits (Qiagen, Valencia, CA, USA). Reverse transcription was carried out with 2 μg total RNA, 8 μL reaction buffer, 2 μL dNTPs, 4.8 μL $MgCl_2$, 4 μL Oligo-dT (10 pmole/L) and 200 U RTase (Promega, Madison, WI, USA) with diethyl pyrocarbonate H_2O in a final volume of 40 μL at 42°C for 1 hour. After heat inactivation, 1 μL cDNA product was used for quantitative real-time polymerase chain reaction (PCR) to determine the expression of tissue inhibitor of metalloproteinase 1 (TIMP-1), TIMP-2 and TIMP-3. Real-time PCR was carried out in a 15 μL reaction volume using ABsolute Blue SYBR Green ROX (Thermo Scientific, Epson, Surrey, UK) with the following primers: iNOS(F:5′CCAACAATACAAGATGACCCTAAG3′; R: 5′GTTGATGAACTCAATGGCATGAG3′; NM_010927.3), Bip(F:5′CTATTCCTGCGTCGGTGTGTTCAA3′; R: 5′GGTTTGCCCACCTCCAATATCAA3′; AJ002387.1), XBP-1(F:5′GAAAGCGCTGCGGAGGAAAC3′; R: 5′GAGGGGATCTCTAAAACTAGAGGC3′; NM_013842), ATF4(F:5′GCCATCTCCCAGAAAGTTT3′; R: 5′AGGTGGGTCATAAGGTTTGG3′; NM_009716), Calr(F:5′AAGAGGACAAGAAGCGTAAA3′; R: 5′ATCAGAATCTACCCCAGATCT3′; NG_029662), IRE1(F:5′GTGTCGTCAGCAGCAGTCTCT3′; R:5′GGGTCCCTGGTCATTGAG3′; AF071777); TIMP-1(F: 5′GGCATCCTCTTGTTGCTATCACTTG3′; R: GTCATCTTGATCTCATAACGCTGG3′; NM_009992.3), TIMP-2 (F: 5′CTCGCTGGAGGACGTTGGAGGAAAGAA3′; R: 5′AGCCCATCTGGTACCTGTGGTT

Table 1
Effects of noni juice on body and liver weight/size changes in experimental rats.

Group	BW (g)	LW (g)	LW/BW (%)
Control	453.37 ± 6.73a	10.46 ± 0.71b	2.37 ± 0.04c
TAA	335.55 ± 6.59c	12.57 ± 1.08a	3.51 ± 0.06a
TAA + NJ-L	351.88 ± 2.03b,c	11.51 ± 0.43a	3.15 ± 0.06b
TAA + NJ-M	359.54 ± 5.79b	11.50 ± 0.48a	3.27 ± 0.08b
TAA + NJ-H	363.32 ± 4.27b	11.91 ± 0.37a	3.23 ± 0.06b

The data are presented as mean ± SEM ($n = 10$). Mean values with different letters are significantly different ($p < 0.05$).
BW = body weight; LW = liver weight; NJ-H = high-dose noni juice; NJ-L = low-dose noni juice; NJ-M = medium-dose noni juice; TAA = thioacetamide.

CA3′; NM_011594), TIMP-3(F:5′CTTCTGCAACTCCGACATCGTGAT3′; R: 5′CAGCAGGTACTGGTACTTGTTGAC3′; NM_000362), GAPDH(F: 5′-GACCCCTTCATTGACCTCAAC-3′,R: 5′-GGAGATGATGACCCTTTTGGC-3′; NM_007393.2). To avoid amplification of genomic DNA, the primers were placed at the junction of two exons. Semiquantitative real-time PCR was done using GAPDH as an internal control for normalization of other gene expressions.

2.10. Activities of matrix metalloproteinase-2 and -9 in liver tissues

The activities of matrix metalloproteinase (MMP)-2 and MMP-9 in liver tissues were measured by gelatin zymography protease assays as described by Chang et al.[18] Briefly, liver homogenates were prepared with sodium dodecyl sulfate sample buffer without boiling or reduction and subjected to 0.1% gelatin−8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer (40mM Tris−HCl, pH 8.0; 10mM $CaCl_2$ and 0.01% NaN_3) at 37°C for 12 hours. Gels were stained with Coomassie Brilliant Blue R-250, and gelatinolytic activities were detected as clear bands against the blue background.

2.11. Statistical analysis

The values are expressed as the mean ± standard error of the mean. 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. Additionally, all statistical analyses of data were performed using SAS (SAS Institute Inc., Cary, NC, USA).

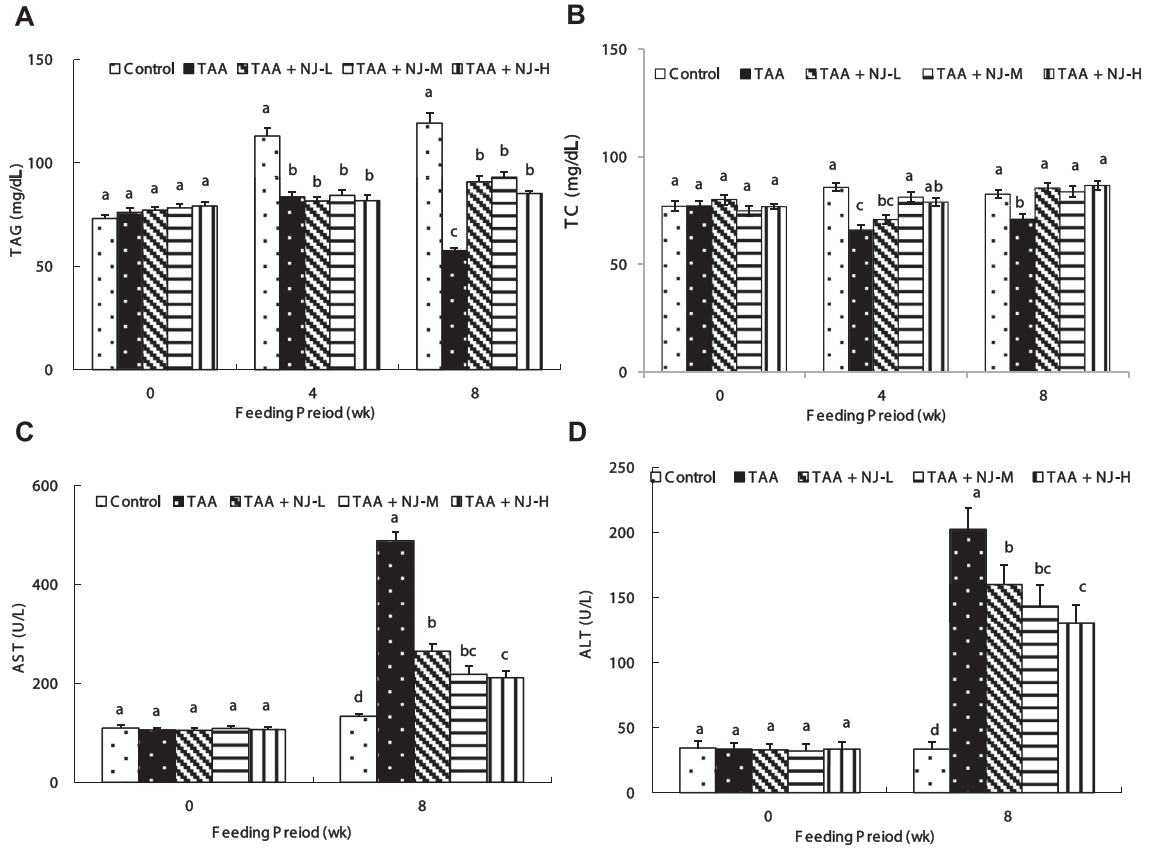

Fig. 1. (A) Serum triacylglycerol; (B) total cholesterol; (C) AST; and (D) ALT levels of the experimental rats. The data are given as mean ± SEM (n = 10). Mean values with different letters on data bars are significantly different ($p < 0.05$). ALT = alanine aminotransferase; AST = aspartate aminotransferase; NJ-H = high-dose noni juice; NJ-L = low-dose noni juice; NJ-M = medium-dose noni juice; TAA = thioacetamide; TAG = triacylglycerol; TC = total cholesterol.

3. Results

3.1. Analysis of bioactivity compounds from NJ

In order to understand the quality of NJ stored at −20°C for 1 year, the total amounts of phenolic acids, flavonoids, condensed tannin, ascorbic acid, and polysaccharides were measured in this study. The amounts of phenol acids, flavonoids, and condensed tannin found were 80.68 ± 3.11 mg gallic acid equivalent/100 mL, 11.55 ± 2.01 mg catechin equivalent/100 mL, and 14.67 ± 0.92 mg catechin equivalent/100 mL, respectively, while there were 21.44 ± 1.36 mg/100mL and 2253.32 ± 56.32 mg/100 mL ascorbic acid and crude polysaccharide, respectively.

3.2. Effects of NJ on body weight and liver weight changes of TAA-treated rats

After 8 weeks of experiments, the body and liver weights of rats among groups were measured; the results are shown in Table 1. TAA treatment significantly decreased the body weight but increased the liver weight and sizes (LW/BW, %) compared to those of the control group ($p < 0.05$). NJ cotreatment ameliorated the increased body weights compared to the TAA-treated group (TAA group vs. TAA + NJ-M and TAA + NJ-H group, $p < 0.05$). Although there were not significant differences in liver weight (g) among the TAA-treated rats ($p > 0.05$), rats treated with TAA were found to have increased liver sizes compared to the control group which was not treated with TAA (TAA group vs. control group, 1:1.48; $p < 0.05$); meanwhile, NJ cotreatment lowered the liver sizes in TAA-treated rats ($p < 0.05$).

3.3. Effects of NJ on serum biochemical parameters, liver lipid and collagen contents, and histopathological analysis of TAA-treated rats

In the present study, serum lipid and liver damage indices (Fig. 1), as well as liver lipid and collagen contents (Fig. 2) of rats treated with TAA were changed when compared to those of the control rats ($p < 0.05$). TAA treatment reduced serum TAG and TC levels ($p < 0.05$) at the 4th and 8th week of observations (Figs. 1A and 1B), but increased AST and ALT values ($p < 0.05$) at the end of experiment (Figs. 1C and 1D).

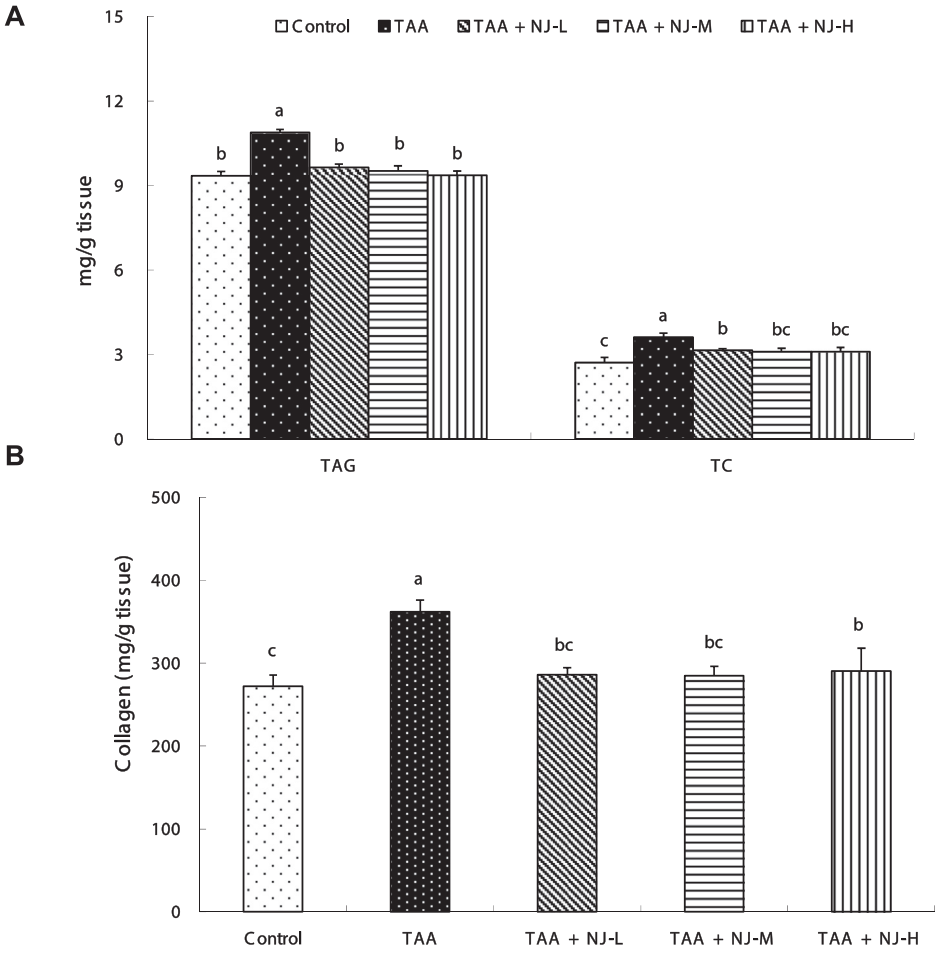

Fig. 2. Liver TAG and TC contents (A), and collagen contents (B) of the experimental rats. The data are given as mean ± SEM (n = 10). Mean values with different letters on data bars are significantly different ($p < 0.05$). NJ-H = high-dose noni juice; NJ-L = low-dose noni juice; NJ-M = medium-dose noni juice; TAA = thioacetamide; TAG = triacylglycerol; TC = total cholesterol.

Meanwhile, liver TAG and TC levels (Fig. 2A), as well as collagen contents (Fig. 2B) were also increased when compared with the control group ($p < 0.05$). However, those elevated values were significantly improved by NJ cotreatment compared to those of the TAA group ($p < 0.05$) (Figs. 1C, 1D, and 2). NJ cotreatment increased serum TAG levels in TAA-treated rats ($p < 0.05$) but the values were still lower than those of the control rats ($p < 0.05$), while NJ cotreatment normalized serum TC levels in TAA-treated rats to that of control rats ($p < 0.05$). As an observation in histopathological examination, H&E staining revealed that TAA treatment caused fibrotic scars with mild inflammatory cell infiltration (Fig. 3A). The histological activity index scores also indicated higher portal and lobular inflammation and periportal necrosis in TAA-treated rats than in the control rats ($p < 0.05$), but only the periportal necrosis levels of TAA-treated rats were ameliorated by NJ cotreatment ($p < 0.05$) (Fig. 3B). Masson's trichrome staining showed the severe collagen accumulation (part of the blue staining) in the TAA-treated groups while NJ cotreatment remarkably reduced the accumulation of those fibrotic tissues (Fig. 3C).

3.4. Effects of NJ on TBARS level, GSH contents, and antioxidant enzymatic activities in livers of TAA-treated rats

It was observed that TAA treatment resulted in an increased hepatic TBARS level and reduced hepatic GSH content as compared to those of the control group ($p < 0.05$), suggesting the impairment of hepatic antioxidant capabilities. Although NJ cotreatment did not alter hepatic TBARS values in TAA-treated rats ($p > 0.05$), there was a tendency toward a higher hepatic content of reduced of GSH in TAA-treated rats with NJ cotreatment, while a significant difference was only observed at medium dosages of NJ (TAA + NJ-M group). Changes of lipid peroxidation and antioxidant capacities in livers are demonstrated in Table 2. Regarding antioxidant enzymatic activities, higher SOD and CAT activities were measured in the TAA-treated rats ($p < 0.05$) compared to the control group, but NJ cotreatment further enhanced those activities in TAA-treated rats ($p < 0.05$). Also, the TAA group had lower hepatic GSH-Px activity ($p < 0.05$) than the control group, but NJ cotreatment normalized this ($p < 0.05$; TAA + NJ-L group), or even resulted in higher GSH-Px activities (TAA + NJ-M and TAA + NJ-H groups) than that of the control group.

3.5. Effect of NJ on mRNA expressions of iNOS, ER stress, and TIMPs, and inflammatory cytokines and MMP-9/MMP-2 activities in livers of TAA-treated rats

After TAA treatment, the mRNA levels of iNOS in the rat livers were substantially upregulated ($p < 0.05$) by approximately 2.4-fold compared with that of the control group (Fig. 4A). However, NJ cotreatment retarded the upregulated iNOS expressions ($p < 0.05$). These downregulated changes represent about a 35% reduction for iNOS in the TAA group

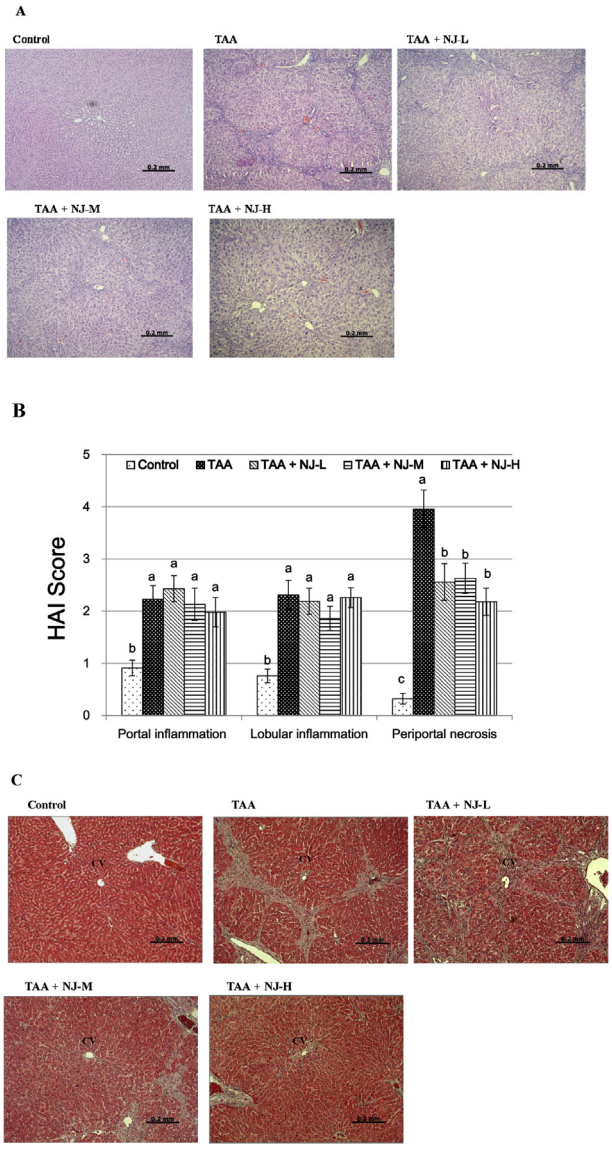

Fig. 3. (A) The H&E staining of representative liver tissues in experimental rats; (B) HAI scores of liver portal, lobular, and periportal necrosis; and (C) Masson's trichrome staining for liver tissues in experimental rats. The data are given as mean ± SEM ($n = 10$). Mean values with different letters on data bars are significantly different ($p < 0.05$). CV = abbreviation for central vein; HAI = histological activity index; H&E = hematoxylin and eosin; NJ-H = high-dose noni juice; NJ-L = low-dose noni juice; NJ-M = medium-dose noni juice; TAA = thioacetamide.

compared to the TAA groups cotreated with NJ. Similarities were detected in gene expressions of BiP (immunoglobulin binding protein or glucose-regulated protein 78-kDa, GRP78), IRE1 (inositol-requiring enzyme 1), and XBP1 (X-box binding protein 1), while the lower ($p < 0.05$) ATF4 (activating transcription factor 4) gene expression was only observed in low dosage NJ supplementation (TAA + NJ-L group) compared to that of the TAA group. However, the increased Calre gene expressions in TAA-treated rats compared to that of the control rats were not influenced by NJ cotreatment ($p > 0.05$; Fig. 4A). Liver TNF-α and IL-1β levels in the TAA-treated rats were significantly higher than those in the control rats

Table 2
Liver peroxidation, antioxidant capacities, and cytokine levels of the experimental rats.

	Control	TAA	TAA + NJ-L	TAA + NJ-M	TAA + NJ-H
Liver lipid peroxidation					
TBARS (nmole MDA eq./mg protein)	0.11 ± 0.00b	0.28 ± 0.01a	0.26 ± 0.01a	0.26 ± 0.01a	0.25 ± 0.01a
Liver antioxidant capacities					
Reduced GSH (nmole/mg protein)	53.95 ± 3.23a	40.77 ± 2.73b	44.07 ± 2.84b	55.52 ± 3.57a	47.50 ± 4.04ab
SOD (munit/mg protein)	17.29 ± 1.12b	19.23 ± 1.80b	25.00 ± 0.92a	26.86 ± 1.29a	28.70 ± 2.83a
CAT (unit/mg protein)	16.59 ± 1.76d	20.47 ± 0.70c	25.24 ± 1.31b	29.57 ± 0.97a	28.73 ± 0.90a
GSH-Px (nmole NADPH oxidized/min/mg protein)	66.08 ± 3.32b	51.01 ± 2.19c	70.85 ± 3.08ab	73.71 ± 2.83a	76.19 ± 1.36a
Cytokine					
TNF-α (pg/mg protein)	3.87 ± 0.26b	5.02 ± 0.14a	4.84 ± 0.26a	4.88 ± 0.29a	4.51 ± 0.22ab
IL-1β (pg/mg protein)	17.66 ± 0.74c	26.05 ± 0.95a	20.62 ± 0.71b	20.58 ± 1.34b	22.83 ± 1.02b

The data are presented as mean ± SEM ($n = 10$). Mean values with different letters are significantly different ($p < 0.05$).
CAT = catalase; GSH = glutathione; IL = interleukin; MDA = malondialdehyde; NJ-H = high-dose noni juice; NJ-L = low-dose noni juice; NJ-M = medium-dose noni juice; SOD = superoxide dismutase; TAA = thioacetamide; TBARS = thiobarbituric acid reactive substances; TNF-α = tumor necrosis factor.

($p < 0.05$), but only IL-1β levels in TAA-treated rats were decreased by NJ supplementation (Table 2). Among TIMP-1, TIMP-2, and TIMP-3 mRNA expressions, hepatic TIMP-1 and TIMP-3 gene expressions were increased ($p < 0.05$) after 8-week injury compared with those of the control group, while TIMP-2 gene expressions were not different among groups ($p > 0.05$; Fig. 4B). However, NJ cotreatment down-regulated hepatic TIMP-1 and TIMP-3 expressions ($p < 0.05$). For detecting the degrees of inhibition of MMP-2 and MMP-9 activities by TIMPs in this study, the zymography technique was applied (Fig. 4C). The MMP-2 and MMP-9 activities in the control group were very low, but TAA treatment dramatically increased those activities. NJ cotreatment significantly decreased activities of those two enzymes in TAA-treated rats ($p < 0.05$), with a larger decrease in relative activities of MMP-9 than in MMP-2.

4. Discussion

Growing understandings of the pathophysiology behind liver fibrosis has contributed to the development of agents that could potentially inhibit and reverse the fibrotic process in livers. In the present study, the damage and inflammation of hepatocytes were observed and measured by serum biochemical values, liver cytokines, and H&E and Masson's trichrome stainings. Our results showed that an application of 100 mg TAA/kg BW three times weekly successfully induces chronic liver fibrosis in rats, which also confirmed the observation from a previous report.[19] According to the histopathological observations, NJ cotreatment showed amelioration of liver damage in TAA-treated rats. It has been reported that the hepatoprotection of silymarin on TAA-induced chronic liver damage is attributed to downregulation of hepatic MMP-2, MMP-13, TIMP-1, TIMP-2, activator protein 1, Kruppel-like factor 6, transforming growth factor beta 1, alpha smooth muscle actin, and collagen alpha 1.[3] Based on our results, there was a remarkable reduction in liver fibrotic scars of TAA-treated rats cotreated with NJ (Fig. 3C), which warrants clarifying the protective molecular mechanisms of NJ on the liver of TAA-treated rats. Furthermore, serum ALT and AST values are also important criteria for the evaluation of liver injuries where the TAA induction results in significant increases.[19] Rats intoxicated with TAA experienced hepatic injury evidenced by significant changes (Fig. 1) in serum liver biomarkers when compared to control rats. Cotreatments of low, medium, and high dosages of NJ in TAA-treated rats significantly lowered the ALT level by 20.01%, 29.2%, and 35.7%, respectively, as well as the AST level by 45.72%, 55.26%, and 56.65%, respectively; this is compared with those without NJ, indicating a potential hepatoprotection effect of NJ supplementation against TAA-induced liver damage (Figs. 1C and 1D).

In addition, the TAA-treated groups showed a significant reduction in body weight with significantly increased liver weights and sizes compared to control rats, but NJ cotreatment reversed those defects (Table 1). The reduction in body weights could be attributed to the toxic effect of TAA throughout the period of the experiment.[3,19] Moreover, TAA altered fatty acid composition in tissues, thus decreasing fatty acid biosynthesis in the liver and lowering serum TAG levels.[20] It has been reported that an improvement in serum lipids and oxidative status in high-cholesterol/fat dietary hamsters treated with NJ is highly related to the regulation of lipid homeostasis by the phytochemicals in NJ.[9] Pallottini et al[20] also indicated that TAA reduces the 3-hydroxy-3-methylgltaryl coenzyme A reductase activity, which may explain the lower serum TC level observed in rats treated with TAA (Fig. 1). Furthermore, the TAA group strongly showed a significant elevation of liver TC and TAG levels in comparison to NJ-cotreated groups (Fig. 2). Fatty acid accumulation also leads to the induction of ER stress and ROS formation, which again hastens the hepatic injury.[21] TAA-induced liver fibrosis is caused by free radical-mediated lipid peroxidation.[19] Lipid peroxidation occurs during the processes of liver fibrosis and inflammation. ROS generation, mitochondrial dysfunction, and antioxidant insufficiency have been reported to advance the development of liver cirrhosis.[12,19] Thus, oxidative stress also triggers production of inflammatory cytokines, causes inflammatory and fibrogenic responses, and is recognized as being of major importance in the progression of this disease. The current study demonstrated that TAA-treated rats exhibit an increase in hepatic TBARS values and a decrease in the

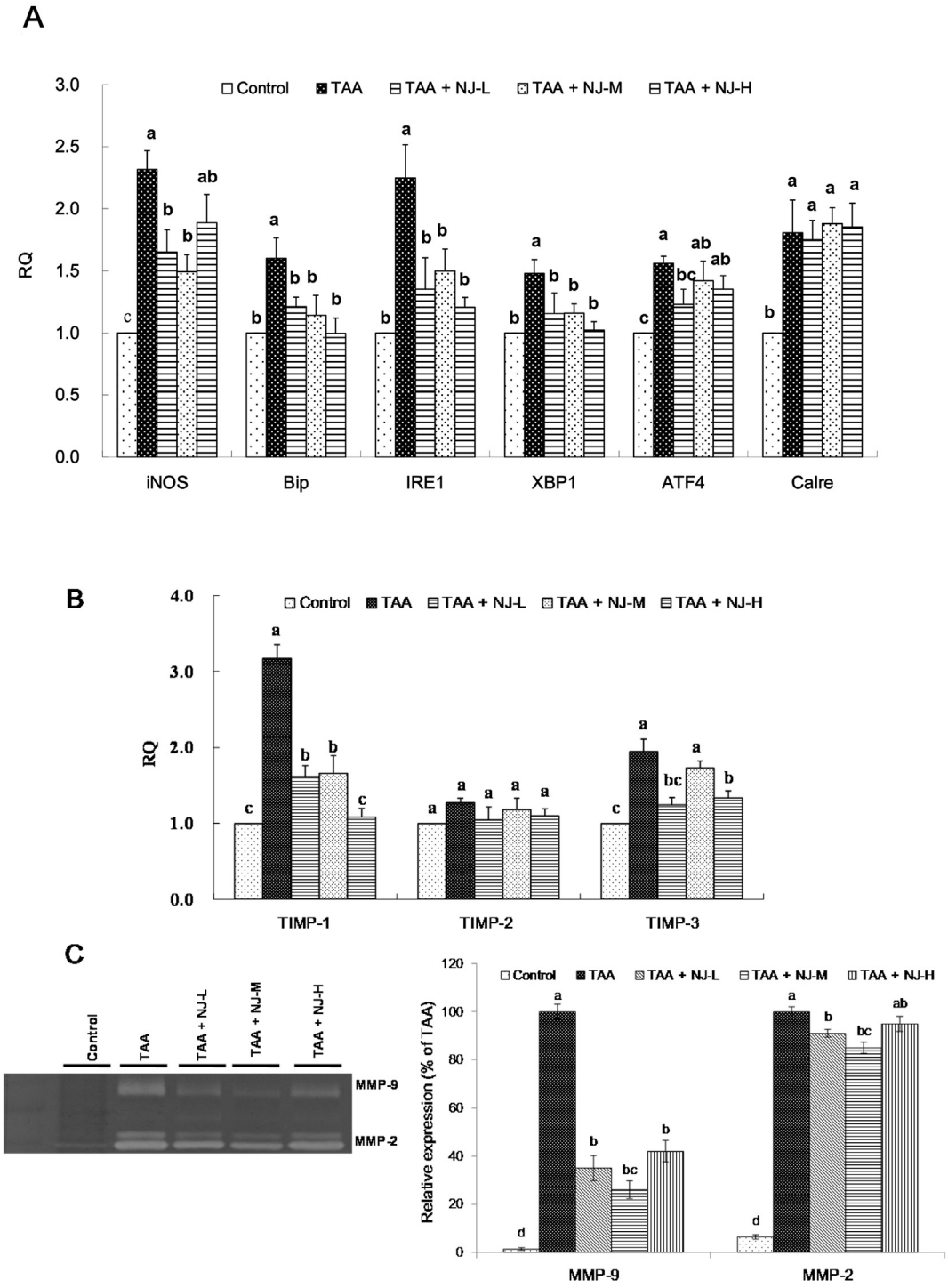

Fig. 4. The gene expressions of (A) the endoplasmic reticulum stress pathway; (B) TIMPs; and (C) MMP-2 and MMP-9 activities in livers of the experimental rats. The data are given as mean ± SEM ($n = 10$). Mean values on data bars in each target gene with different letters were significantly different ($p < 0.05$). NJ-H = high-dose noni juice; NJ-L = low-dose noni juice; NJ-M = medium-dose noni juice; MMP = matrix metalloproteinase; RQ = relative quantification; TAA = thioacetamide; TIMP = tissue inhibitor of metalloproteinase.

reduced GSH content as compared to control rats, suggesting the impairment of hepatic antioxidant capabilities. Our results showed that NJ supplementation can decrease the liver TBARS values but increase the content of reduced GSH in the liver, as well as SOD, CAT, and GSH-Px activities in TAA-treated rats (Table 2). Those benefits should result from the bioactive compounds, i.e., polyphenols, polysaccharides, Se, Mn, etc., in NJ.[10] Also, NJ cotreatment effectively reduced serum ALT and AST levels (Fig. 1), hepatic fibrosis scores (Fig. 3B), the gene expressions of iNOS, Bip, IRE1, XBP-1, and ATF4 in livers (Fig. 4A), and hepatic IL-1β contents (Table 2) and collagen accumulation (Fig. 2B) in TAA-treated

rats. Therefore, an antiinflammatory or antifibrosis effect of NJ against a TAA induction could correspond highly to the reduction of liver oxidative levels. Oxidative stress triggers ER stress and unfolded protein response, however, excessive or prolonged ER stress decreases the mitochondrial membrane potential, limits bioenergetics changes, and fosters the generation of ROS, ultimately inducing apoptosis.[22] Naturally fermented NJ contains plenty of polysaccharides, polyphenols, and some trace minerals (Zn, Mn, and Se).[10] Natural polysaccharides, largely found in fruits and vegetables, have been confirmed to play a vital role as free radical scavengers.[23] It has also been demonstrated that polysaccharides and polyphenols are synergistic in the reduction of serum leptin levels and antiinflammatory activities.[24] Trace minerals Mn and Se are cofactors for SOD and GSH-Px, respectively. Zn has antioxidant and anticancer activities and helps to reduce cardiovascular disease.[25] Moreover, activated HSCs can downregulate tissue TIMPs, leading to a hypothesis that matrix degradation is inhibited during progressive fibrosis.[26] Our results showed that TAA induces proinflammatory responses, as evidenced by increased ROS production, cytokine release (TNF-α and IL-1β; Table 2), and matrix degrading enzyme activation (MMP-9 and MMP-2; Fig. 4C).The TIMP-1 inhibition may not be maximal and MMP-mediated degradation still occurs in remodeling during progressive fibrosis.[27] Hence, NJ cotreatment exerts hepatoprotective effects against TAA damage, possibly via downregulation of MMP-2 and MMP-9 activities and upregulation of TIMP-1 and TIMP-3 (Figs. 4B and 4C). Taken together, these data strongly suggest that the ameliorative effect of NJ on liver fibrotic tissues is potentially due to a regulation of MMP-9-mediated degradation, where an active MMP-9 could be inhibited by an interaction with TIMP-1and TIMP-3 *in vivo*.

In conclusion, the current study presents solid evidence showing the hepatic oxidative stress and damage, along with lipid accumulation, inflammation, ER stress and its associated cell death, fibrotic responses, and consequently hepatic dysfunctions in TAA-treated rats. It also demonstrated that supplementing with naturally fermented NJ in TAA-treated rats alleviates these pathological changes, including ER stress (Fig. 4), lipid accumulation (Fig. 2), inflammation (Table 2), and fibrosis development (Figs. 3 and 4).Therefore, it is speculated that the synergistic effect of bioactive compounds (polyphenol, polysaccharide, Zn, Mn, and Se) in this naturally fermented NJ may offer the liver protection against the TAA intoxication. In summary, naturally fermented NJ can attract considerable attention in the functional-food market for its ameliorative effect against chronic liver fibrosis.

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