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Research Paper

Inhibitory effect of phytoglycoprotein (24 kDa) on hepatocarcinogenesis in *N*-nitrosodiethylamine-treated ICR mice

Jin Lee[ab] and Kye-Taek Lim[a]

Molecular Biochemistry Laboratory, [a]Biotechnology Research Institute and [b]Center for the Control of Animal Hazards Using Biotechnology (BK21), Chonnam National University, Gwang-ju, South Korea

Abstract

Objectives Hepatocellular carcinoma (HCC) is becoming one of the most prominent types of cancer in the world. For a long time in Korea *Zanthoxylum piperitum* DC (ZPDC) has been used in folk medicine to cure several cancers and inflammation. This study was designed to investigate whether ZPDC glycoprotein protected liver tissues against hepatocarcinogenic compounds such as *N*-nitrosodiethylamine (DEN).

Methods To study the chemopreventive effect of ZPDC glycoprotein on hepatocarcinogenesis, ICR mice were injected intraperitoneally with DEN (50 mg/kg) for four weeks. We evaluated the indicators of liver tissue damage (the activity of lactate dehydrogenase (LDH) and alanine aminotransferase (ALT), and thiobarbituric acid-reactive substances (TBARS)), antioxidative enzymes (activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx)), hepatocarcinogenic indicator (heat shock protein (HSP) 70) and hepatocarcinogenic signals (activity of nuclear factor (NF)-κB, cyclooxygenase (COX)-2, and matrix metalloproteinase (MMP)-9) using biochemical methods and immunoblot analysis.

Key findings The results obtained from this study revealed that ZPDC glycoprotein (20 mg/kg) decreased the levels of LDH, ALT, and TBARS, whereas the activity of SOD and GPx increased in the DEN-treated ICR mice. With respect to the hepatocarcinogenic indicator and hepatocarcinogenic signals, HSP70, NF-κB, COX-2, and MMP-9 activity decreased.

Conclusion The findings suggested that ZPDC glycoprotein prevented damage to liver tissue caused by DEN in the experimental mouse model.

Keywords heat shock protein 70; matrix metalloproteinase 9; nuclear factor-κB; *N*-nitrosodiethylamine; *Zanthoxylum piperitum* DC glycoprotein (24 kDa)

Introduction

Hepatocellular carcinoma (HCC) is one of the most fierce solid tumours with global annual diagnosis exceeding one million new cases and ranking as the third leading cause of cancer death.[1] Toxic industrial chemicals, air, water pollutants, food additives and fungal toxins are major sources of hepatocarcinogenesis.[2] *N*-nitrosodiethylamine (DEN) is a representative chemical of a family of carcinogenic *N*-nitroso compounds which are categorized as carcinogenic chemicals. DEN is a potent hepatocellular carcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals, occupational settings, cosmetics, agriculture chemicals and pharmaceutical agents.[3] It has been shown that on primary metabolic activation, DEN produces the promutagenic adducts, O^6-ethyldeoxyguanosine and O^4- and O^6-ethyldeoxythymidine, that can produce DNA-chain damage, depurination or binding to DNA and often leads to the generation of a miscoding gene sequence, paving the way to initiation of liver carcinogenesis.[4]

Lipid peroxidation and subsequent autocatalytic disruption of membranes have been implicated in the pathogenesis of a number of diseases and clinical conditions including diabetes, atherosclerosis, Parkinson's disease, Alzheimer's disease, various chronic inflammatory conditions, cancer and many more.[5] Superoxide dismutase (SOD) and glutathione peroxidase (GPx), which consists of tripeptide, constitute mutually a supportive team of defence against reactive oxygen species (ROS). They play a critical role as a marker of chemoprevention due to their antioxidant and detoxification properties.[6] SOD is a metalloprotein that catalyses the dismutation of superoxide radicals.

Correspondence: Kye-Taek, Molecular Biochemistry Laboratory, Biotechnology Research Institute, Chonnam National University, 300 Yongbong-Dong, Gwang-ju 500-757, South Korea.
E-mail: Ktlim@chonnam.ac.kr

It has been reported that the process of chronic inflammation leads to stressful conditions. Such stressful stimuli induce ubiquitous molecules like heat shock proteins (HSPs), thereby contributing to hepatocarcinogenesis.[7] One of the prominent members in the HSP family is HSP70. Emerging evidence strongly supports the link between the expression of HSP70 and the oncogenic potential of tumour cells, indicating that HSP70 could be an attractive target for not only cancer therapy but also chemoprevention. Nuclear factor (NF)-κB is involved in similar biological promotion such as cell survival, inflammation, angiogenesis, progression, and metastasis. Also, the abnormal activity of NF-κB modulates the transcription and expression of genes, and plays an important role in the development of HCC.[8,9] Cyclooxygenase (COX) is the rate limiting enzyme which is involved in the conversion of arachidonic acid to prostaglandin H_2 (PGH_2). Various studies have reported that an increase in COX-2 expression may contribute to liver damage and carcinogenesis in distinct animal models, as well as to HCC in humans.[10–13] Matrix metalloproteinases (MMPs) are a family of zinc-containing endopepidases and their endogenous tissue inhibitors (TIMPs).[14] Among MMPs, MMP-9 is secreted as a proenzyme, having a molecular weight of 92 kDa, and can be activated *in-vitro* by organomercurials, trypsin and α-chymotrypsin, and in-vivo by catepsin G. Also, MMP-9 is closely related to hepatocellular carcinomas and abundantly expressed in various malignant tumours. Especially, it plays a critical role in tumour invasion, metastasis and angiogenesis in liver cancer. To find any kind of MMP inhibitor is one of the good strategies for the prevention of initiation, tumour progression, metastasis, and angiogenesis in several cancers.

Generally, glycoproteins can be divided into two categories. The first is P-glycoproteins, which are defined as plasmic glycoprotein on the intra- or extra-cellular membrane and act as multidrug resistance. The second is phytoglycoproteins, which originate from herbal plants. Both glycoproteins play a critical role in the living system having bioactivity. Interestingly, it has been reported that the activity of P-glycoprotein can be lost or inhibited by either antibiotics or pepper extract.[15,16] On the other hand, natural compounds such as phytoglycoproteins have themselves a utility in the prevention or healing of damage to the liver by certain chemicals which initiate the development of cancer or tumour, because they have less cytotoxic activity and side effects than artificial synthetic drugs. Chemoprevention is defined as the use of natural products to inhibit the development of invasive cancer either by blocking DNA damage or by arresting the progression of premalignant cells.[17] In Asian countries, herbal plants have been used for many years in traditional folk medicine (chemoprevention) for healing several types of cancers. It has been reported that *Zanthoxylum piperitum* DC (ZPDC) is one of the well-known spices, used to produce a fresh flavour and to avoid unpleasant fishy and meaty odours.[18] In Korean folk medicine it has been used to heal diarrhoea and abdominal pain.[19] Recently, we isolated glycoprotein (24 kDa) from ZPDC fruits, which consisted of a carbohydrate moiety (18%) and a protein moiety (82%). We reported that the ZPDC glycoprotein had strong characteristics relating to antioxidation, cytoprotection and anti-inflammation.[20] Most glycoproteins originating from plants have a modulation characteristic relating to immune-related factors such as anti-inflammation, anti-apoptosis and antitumour, and so we assumed that ZPDC glycoprotein could probably prevent the hepatocarcinogenesis in an animal model.[21,22] However, it has not been reported that ZPDC glycoprotein prevented hepatocarcinogenesis by DEN.

Therefore, the aim of this study was to investigate whether ZPDC glycoprotein could contribute to the inhibition of hepatocarcinogenesis caused by DEN. We evaluated indicators of liver tissue damage (lactate dehydrogenase (LDH), alanine aminotransferases (ALT) and thiobarbituric acid-reactive substances (TBARS)), antioxidative enzymes (SOD and GPx), hepatocarcinogenic indicator (HSP70), and hepatocarcinogenic signals (NF-κB, COX-2, and MMP-9) after treatment with ZPDC glycoprotein in the DEN-treated ICR mice using biochemical methods and immunoblot analysis.

Materials and Methods

Chemicals

All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). DEN, benzylpenicillin and streptomycin were obtained from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest analytical grade available.

Preparation of ZPDC glycoprotein

Fruits of *Z. piperitum* DC (ZPDC) were obtained in October 2006 from Na-Ju in the Chonnam province of South Korea. Glycoprotein was isolated and purified as described previously.[20] The ZPDC was cut into small pieces and soaked in 99% ethanol at 4°C for three months in a dark basement. The extract was filtered through Whatman filter paper (No. 2) and then evaporation was performed to remove the 99% ethanol using a rotary evaporator (B465; Buchi, Flawil, Switzerland). After the evaporation of ethanol, the remaining solution was freeze-dried (SFDS06; Sam won, Seoul, Korea) to make a fine powder. The dried powder (5 g) was re-dissolved in distilled water and precipitated with 80% ammonium sulfate to obtain a protein. Following these steps, the sample was dialysed (membrane with molecular weight cut-off 6000–8000 Da) to obtain protein with a molecular weight of more than 8 kDa and then centrifuged with using a Microcon (Millipore; molecular weight cut-off 30 000) in accordance with the manufacturer's protocol (Amicon Inc., MA, USA). The final amount of ZPDC glycoprotein was 3.5 mg (0.07%) from the initial amount (5 g) of dried ZPDC powder. To characterize the sample, SDS-PAGE was carried out loading the sample of protein (1 mg/10 μl) containing 0.1% SDS, using a 18% polyacrylamide mini-gel and Mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA, USA) at 110 V, 30 mA for 2.5 h. The ZPDC glycoprotein on the gel was stained with Schiff's reagent, which is a specific staining reagent for glycoprotein through a redox-reaction.[23] The sample of glycoprotein on the gel revealed one band with molecular weight 24 kDa, consisting of carbohydrate (18%) and protein (82%).[20]

Experimental design

Male mice (ICR; 3-weeks old) were purchased from Daehan Lab (Animal Research Center Co., Ltd, DaeJeon, Korea). Animals were maintained in a clean room at a temperature between 23 ± 2°C with a 12 h light/dark cycle, lights (an illumination intensity of 150–300 lux) on 0700 h and lights off 1900 h. The relative humidity was 55 ± 15% with air ventilation frequencies of 15–20 times/h. Animals were housed according to Guiding Principles in the Use of Animal in Toxicology, adopted by the Society of Toxicology in 1989 at the Experimental Animal Room of College of Veterinary Medicine, Chonnam National University (CNU). The protocol of the animal study was approved by the Animal Care and Use Committee of the College of Veterinary Medicine, CNU. All mice were fed a commercial diet with water freely available and were kept for at least one week before the experiments. The body weight and food intake of each group were recorded once weekly.

The mice were divided into five groups. Group 1, control (($n = 6$), was fed a commercial diet with water freely available. Group 2 mice ($n = 6$) were orally given 20 mg/kg ZPDC glycoprotein alone daily for five weeks. Group 3 ($n = 6$) received intraperitoneal injections of 0.1 ml saline containing 50 mg/kg DEN once a day for four weeks. The mice in groups 4 and 5 (both groups $n = 6$) were treated with ZPDC glycoprotein (10 or 20 mg/kg, respectively) daily for five weeks and simultaneously treated with 50 mg/kg DEN once a day for four weeks. After week 5, blood from the mice was collected through a cardiac puncture under diethyl ether anaesthesia and centrifuged at 10 000*g* for 5 min at 4°C. The supernatant was separated and stored at −70°C to measure the levels of LDH, ALT and TBARS. Mouse blood was used to measure ALT, LDH, and TBARS after treatment with DEN or DEN under pretreatment with ZPDC glycoprotein. Liver tissues were used to assess activity of antioxidant enzymes (SOD and GPx), HSP70, NF-κB, COX-2 and MMP-9. For determining toxicity, relative values of body and liver weights were calculated as shown in the following equations:

$$\text{Relative value of BW} = \text{BWA (g)}/\text{BWB (g)} \times 100 \quad (1)$$

$$\text{Relative value of LW} = \text{LW (g)}/\text{BW (g)} \times 100 \quad (2)$$

Where BWB is the body weight before administration, BWA is the body weight after administration, LW is the liver weight and BW is the body weight.

Thiobarbituric acid reactive substances assay

Lipid peroxidation was estimated by the amount of TBARS in plasma according to the method of Ohkawa *et al*.[24] One volume of sample was mixed thoroughly with two volumes of stock solution 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 M HCl. The mixture was heated for 30 min in a boiling water bath. After cooling, the flocculate precipitate was removed by centrifugation at 1000*g* for 10 min and the absorbance of the sample was used as the standard. Data are expressed as a percent of the control.

Lactate dehydrogenase and alanine aminotransferase assay

The levels of LDH and ALT in mouse serum were measured according to the method of Bergmeyer and Bernt.[25,26] The activity of LDH and of ALT was measured as the rate of loss of β-NADH absorption at 340 nm for 2 min.

Assay of antioxidant and glutathione-metabolizing enzymes

SOD activity was determined by the method of Kakkar *et al*.[27] in which inhibition of the formation of NADPH-phenazine methosulphate nitroblue tetrazolium formazone was measured spetrophotometrically at 560 nm. GPx was assayed by the method based on the reaction between glutathione remaining after the action of GPx and 5,5′-dithiobid-2-nitrobenzoic acid to form a complex that absorbs maximally at 412 nm.[28]

Preparation of nuclear and whole protein extracts

Liver tissues were homogenized with a Dounce homogenizer in 1 ml buffer A (in mM: 10 HEPES (pH 7.9), 10 KCl, 0.1 EDTA, 0.1 EGTA, 1 DTT, 0.5 PMSA, 1.5 $MgCl_2$, and 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany). The homogenate was centrifuged at 200*g* for 30 s at 4°C. The supernatant was transferred to a clean tube, incubated on ice for 5 min and centrifuged again at 5000*g* for 10 min at 4°C, to separate cytosolic and nucleic protein extracts. For immunoblotting of NF-κB, the pelleted nuclear protein extract were resuspended in 200 µl buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM MgCl2 and 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany). After incubation on ice for 30 min, the tubes were shaken and centrifuged at 12 000*g* for 1 h at 4°C to yield nuclear extract.

To prepare whole extract for immunoblotting of HSP70, COX-2 and MMP-9, liver tissues were homogenized in ice-cold lysis buffer (in mM: 150 NaCl, 0.5% Triton-X 100, 50 Trix-HCl (pH 7.4), 20 EGTA, 1 DTT, 1 Na_3VO_4 and protease inhibitor cocktail tablet). Lysates were centrifuged at 14 800*g* for 30 min at 4°C. The amount of protein was measured by the method of Lowry *et al*.[29] and the cellular proteins were stored at −70°C before use.

Immunoblot analysis

Either nuclear or whole protein extract was isolated from liver tissue as described above. These sample proteins were separated using a 10% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a Mini-PROTEAN II electrophoresis cell (Bio-Rad). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h at room temperature in TBS-T solution (in mM: 10 Tris-HCl, pH 7.6, 150 NaCl, and 0.1% (v/v) Tween-20) containing 5% nonfat dry milk. The membranes were subsequently incubated for 2 h at room temperature with primary antibodies (HSP70, NF-κB p50, and NF-κB p65, COX-2, MMP-9, and α-tubulin) in TBS-T solution. After washing three times with TBS-T, the

Table 1 Effect of *Zanthoxylum piperitum* DC glycoprotein on *N*-nitrosodiethylamine-treated changes in the levels of lactate dehydrogenase and alanine aminotransferase in liver of mice

Treatment	Relative weight (%)			
	Body	Liver	LDH (%)	ALT (U/l)
Control	100 ± 2.5	4.5 ± 0.4	100.0 ± 2.7	27.3 ± 2.7
ZPDC glycoprotein (20 mg/kg, BW)	105 ± 1.2	4.8 ± 1.1	100.3 ± 2.4	28.0 ± 2.1
DEN (50 mg/kg, BW)	83 ± 2.5#	8.2 ± 0.1#	157.4 ± 3.47#	91.8 ± 5.8#
DEN + ZPDC glycoprotein (10 mg/kg, BW)	91 ± 1.7	6.5 ± 0.7	140.5 ± 2.57	71.8 ± 2.4
DEN + ZPDC glycoprotein (20 mg/kg, BW)	98 ± 1.1*	5.8 ± 0.7*	117.9 ± 1.14*	27.5 ± 2.8*

Data represented the mean ± SD ($n = 6$). #$P < 0.05$ compared with control; *$P < 0.05$ compared with *N*-nitrosodiethylamine (DEN) treatment alone. ALT, alanine aminotransferase; BW, body weight; LDH, lactate dehydrogenase ; ZPDC, *Z. piperitum* DC.

membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG (1 : 10 000; Cell Signaling Technology, MA, USA) in TBS-T solution. The resulting protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, UK). The results of the immunoblot assay were calculated by way of relative intensity using Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD, USA).

Statistical analysis

All experiments were carried out in triplicate and data were expressed as means ± SE. All data were analysed using a Kruskal-Wallis test (individual difference between treatments being statistically identified using Dunn's test). Differences were considered to be significant at $P < 0.05$.

Results

Inhibitory effect of ZPDC glycoprotein on lactate dehydrogenase and alanine aminotransferase levels in DEN-treated ICR mice

DEN treatment significantly decreased body weight and increased liver weight, compared with the control. However, ZPDC glycoprotein reversed the body weight loss and liver swelling induced by DEN to the levels of the control group (Table 1). The levels of LDH and ALT were measured in the plasma to evaluate hepatic tissue damage (Table 1). The results indicated that ZPDC glycoprotein (20 mg/kg) did not show a significant hepatotoxicity. Serum LDH and ALT levels in the DEN-treated groups were higher than those in the nontreated groups throughout the experiment. Administration of ZPDC glycoprotein significantly reduced the increased plasma LDH and ALT levels.

Inhibitory effect of ZPDC glycoprotein on lipid peroxidation in DEN-treated ICR mice

TBARS level was assessed as an indicator of lipid peroxidation. DEN treatment significantly increased the level of TBARS in the plasma. As shown in Figure 1, when ICR mice were treated with DEN, TBARS levels significantly increased 1.39-fold compared with the control. However, when ZPDC glycoprotein 10 or 20 mg/kg was administered to ICR mice in the presence of DEN, TBARS level significantly decreased

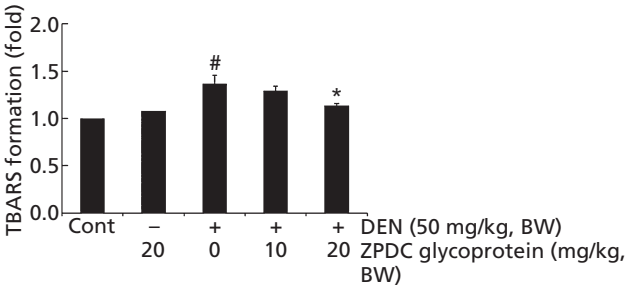

Figure 1 Inhibitory effect of *Zanthoxylum piperitum* DC glycoprotein on lipid peroxidation in *N*-nitrosodiethylamine-treated ICR mice. Lipid peroxidation was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust[30]. All data are the means ± SE from triplicate experiments ($n = 6$), separately. #$P < 0.05$ significant difference between treatments and the control (Cont). *$P < 0.05$ significant difference between *N*-nitrosodiethylamine (DEN) treatment alone and the *Z. piperitum* DC (ZPDC) glycoprotein treatment in the presence of DEN. BW, body weight.

0.07- and 0.08-fold, respectively, compared with levels in mice treated solely with DEN.

Effect of ZPDC glycoprotein on the activity of antioxidant enzymes (SOD and GPx) in DEN-treated ICR mice

The hepatic antioxidant enzyme activity (SOD, GPx) is shown in Figure 2. The activity of SOD and GPx in DEN-treated mice decreased compared with the control. Interestingly, pretreatment with ZPDC glycoprotein resulted in a significant increase in SOD and GPx activity in the DEN-treated group.

Inhibitory effect of ZPDC glycoprotein on expression of HSP70 in DEN-treated ICR mice

We evaluated the impact of the ZPDC glycoprotein on the expression of HSP70 as one of the tumour markers. As shown in Figure 3, the expression of HSP70 was markedly increased by DEN. However, the treatment with ZPDC glycoprotein in the presence of DEN resulted in a significant decrease in the activity of HSP70. For instance, the relative intensity of HSP70 increased 3.49-fold in the presence of DEN, compared with the control. However, the treatment with ZPDC glyco-

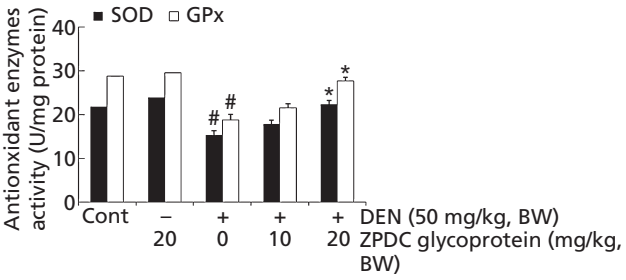

Figure 2 Effect of *Zanthoxylum piperitum* DC glycoprotein on activity of antioxidant enzymes in *N*-nitrosodiethylamine-treated ICR mice. The activity of hepatic superoxide dismutase (SOD) and glutathione peroxidase (GPx) in liver were measured at 560 and 412 nm, respectively. All data are the means ± SE from triplicate experiments ($n = 6$), separately. #$P < 0.05$ significant difference between treatments and the control (Cont). *$P < 0.05$ significant difference between *N*-nitrosodiethylamine (DEN) treatment alone and the *Z. piperitum* DC (ZPDC) glycoprotein treatment in the presence of DEN. BW, body weight.

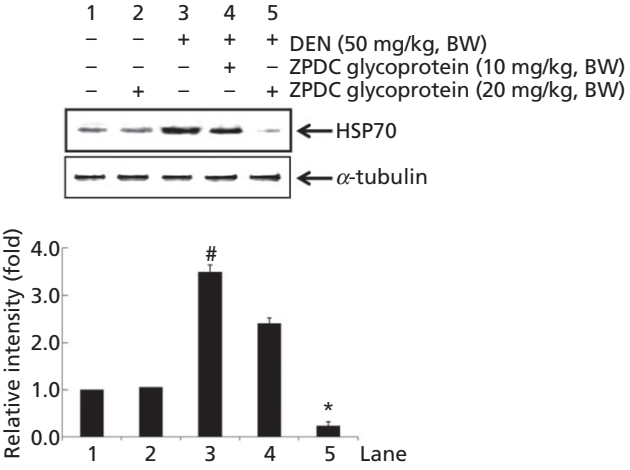

Figure 3 Inhibitory effect of *Zanthoxylum piperitum* DC glycoprotein on expression of heat shock protein 70 in *N*-nitrosodiethylamine-treated ICR mice. The relative intensities of bands obtained from Western blot were calculated using Scion Imaging Software. All data are the means ± SE from triplicates, separately. #$P < 0.05$ significant difference between *N*-nitrosodiethylamine (DEN) treatment alone (lane 3) and the control (lane 1). *$P < 0.05$ significant difference between DEN treatment alone and the *Z. piperitum* DC (ZPDC) glycoprotein treatment in the presence of the DEN (lane 5). BW, body weight; HSP70, heat shock protein 70.

protein (10 or 20 mg/kg) in the presence of DEN diminished HSP70 0.30- and 0.93-fold, respectively, compared with DEN treatment alone.

Inhibitory effect of ZPDC glycoprotein on expression of NF-κB (p50 and p65) in DEN-treated ICR mice

To evaluate the impact of the ZPDC glycoprotein on downstream hepatocyte signalling, we examined the effect of ZPDC glycoprotein on the activation of NF-κB (p50 and p65) using the Western blotting method. When the cells were treated with DEN, the activity of NF-κB markedly increased.

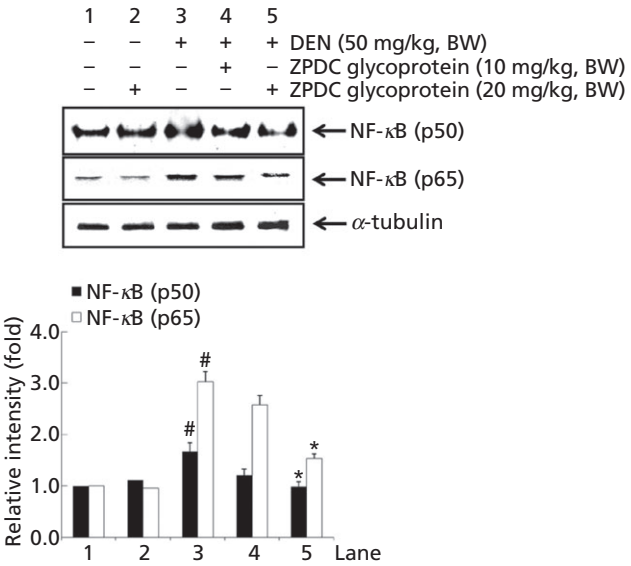

Figure 4 Inhibitory effect of *Zanthoxylum piperitum* DC glycoprotein on expression of nuclear factor-κB (p50 and p65) in *N*-nitrosodiethylamine-treated ICR mice. The relative intensities of bands obtained from Western blotting were calculated using Scion Imaging Software. All data are the means ± SE from triplicates, separately. #$P < 0.05$ significant difference between *N*-nitrosodiethylamine (DEN) treatment alone (lane 3) and the control (lane 1). *$P < 0.05$ significant difference between DEN treatment alone and the *Z. piperitum* DC (ZPDC) glycoprotein treatment in the presence of the DEN (lane 5). α-tubulin was used as internal standard. BW, body weight.

However, the treatment with ZPDC glycoprotein (10 or 20 mg/kg) in the presence of DEN significantly decreased the activity of NF-κB (p50 and p65) in a concentration dependent manner. For instance, the relative intensities of NF-κB (p50 and p65) increased 1.67- and 3.30-fold in the presence of DEN, compared with the control. However, treatment with ZPDC glycoprotein (20 mg/kg) in the presence of DEN diminished NF-κB p50 and p65 0.40- and 0.50-fold, respectively, compared with DEN treatment alone (Figure 4).

Inhibitory effect of ZPDC glycoprotein on expression of MMP-9 in DEN-treated ICR mice

To determine whether ZPDC glycoprotein inhibited the activity of MMP-9 in the DEN-treated ICR mice, activity of MMP-9 was determined using Western blot analysis. As shown in Figure 5, activity of MMP-9 increased after treatment with DEN, compared with the control. For instance, the relative band-intensity of MMP-9 increased 3.57-fold after treatment with DEN, compared with the control. However, when the cells were treated with DEN in the presence of ZPDC glycoprotein (10 or 20 mg/kg), relative band-intensities were markedly reduced in a dose-dependent manner. That is, the band-intensities of MMP-9 after treatment with DEN significantly decreased 0.10- and 0.38-fold, respectively, in the presence of ZPDC glycoprotein 10 and 20 mg/kg, compared with after treatment with DEN alone.

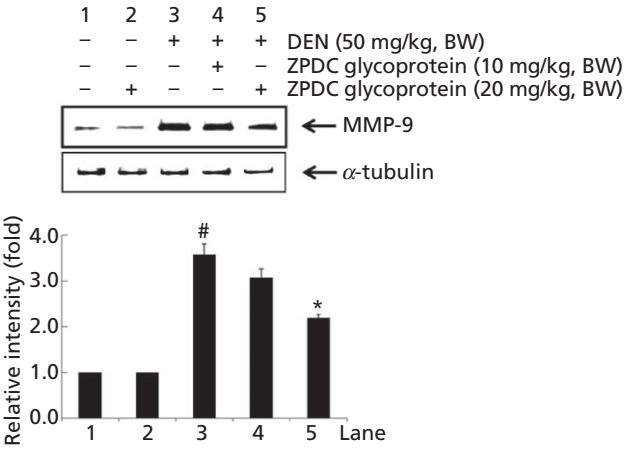

Figure 5 Inhibitory effect of *Zanthoxylum piperitum* DC glycoprotein on expression of matrix metalloproteinase-9 in *N*-nitrosodiethylamine-treated ICR mice. The relative intensities of bands obtained from Western blotting were calculated using Scion Imaging Software. #$P < 0.05$ significant difference between *N*-nitrosodiethylamine (DEN) treatment alone (lane 3) and the control (lane 1). *$P < 0.05$ significant difference between DEN treatment alone and the *Z. piperitum* DC (ZPDC) glycoprotein treatment in the presence of the DEN (lane 5). α-tubulin was used as internal standard. BW, body weight; MMP-9, matrix metalloproteinase-9.

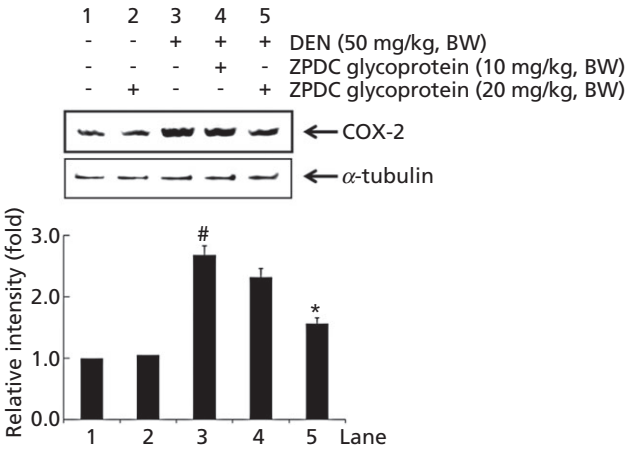

Figure 6 Inhibitory effect of *Zanthoxylum piperitum* DC glycoprotein on expression of cyclooxygenase-2 in *N*-nitrosodiethylamine-treated ICR mice. The relative intensities of bands obtained from the Western blot were calculated using Scion Imaging Software. All data are the means ± SE from triplicates, separately. #$P < 0.05$ represents a significant difference between *N*-nitrosodiethylamine (DEN) treatment alone (lane 3) and the control (lane 1). *$P < 0.05$ significant difference between DEN treatment alone and the *Z. piperitum* DC (ZPDC) glycoprotein treatment in the presence of the DEN (lane 5). α-tubulin was used as internal standard. BW, body weight; COX-2, cyclooxygenase-2.

Inhibitory effect of ZPDC glycoprotein on expression of COX-2 in DEN-treated ICR mice

To determine whether ZPDC glycoprotein inhibited the activity of COX-2 in DEN-treated ICR mice, activity of COX-2 was determined using Western blot analysis. As shown in Figure 6, activity of COX-2 increased after treatment with DEN alone, compared with the control. For instance, the relative band-intensity of COX-2 increased 2.68-fold after treatment with DEN, compared with the control. However, when the cells were treated with DEN in the presence of ZPDC glycoprotein (10 or 20 mg/kg), relative band-intensities were markedly reduced in a dose-dependent manner. That is, the band-intensity of COX-2 after treatment with DEN significantly decreased 0.13- and 0.41-fold, respectively, in the presence of ZPDC glycoprotein 10 and 20 mg/kg, compared with after treatment with DEN alone.

Discussion

Carcinogenesis may arise as a result of chemical or biological damage to normal cells in a multistep process that involves changes at the initiation levels followed by promotion and progression which lead to malignancy.[31] The liver plays a pivotal role in the regulation of physiological processes such as metabolism, secretion and storage. Unfortunately it is a common target for a number of toxicants.[32] One of these such toxicants DEN is well known as a reproducible carcinogen, which induces lesions in mice, malignant tumours in human and is a complete carcinogen that induces HCC.[3] As the liver is the main site for the metabolic biotransformation of DEN, it has been suggested that the uncompromised generation of free radicals and reactive oxygen species in the liver may be responsible for oxidative stress which causes liver damage.[33] The model of DEN-induced HCC is considered one of the most accepted and widely used experimental models to study hepatocarcinogenesis.[3] This study has evaluated the chemopreventive effect of ZPDC glycoprotein against DEN-induced herpatocarcinogenesis.

One major symptom of HCC is weight loss. A significant ($P > 0.05$) weight loss was observed in mice exposed to DEN. Treatment with ZPDC glycoprotein improved body weight which indicated the reversal of chemically induced hepatocarcinogenesis. LDH is a cytosolic enzyme that catalyses the reversible oxidation of L-lactate to pyruvate; its increased activity in serum confirms increased hepatocyte membrane permeability and cellular damage. It has been reported that the level of LDH is strongly correlated with tumour bulk, since a high rate of glycosis takes place in cancerous conditions and glycosis is the only energy producing pathway for malignant cells.[34] The activity of serum enzymes such as ALT normally denotes liver function. Elevations in ALT levels are considered the most sensitive markers in the diagnosis of hepatocellular damage and the loss of functional integrity of the membrane.[35] Elevated serum ALT levels correlate with a high incidence of HCC development in patients with chronic hepatitis. As shown in Table 1, the ZPDC glycoprotein inhibited elevated serum enzymes (LDH and ALT) in DEN-induced HCC. It has been reported that elevated levels of lipid peroxidation (TBARS) in the liver are considered one of the basic mechanisms of tissue damage caused by free radicals and that they act as an important causative factor in carcinogenesis.[36] The results obtained from this study showed that DEN treatment significantly increased TBARS levels, whereas ZPDC glycoprotein (20 mg/kg) normalized the TBARS levels induced by DEN. A possible explanation for this is that the

ZPDC glycoprotein (20 mg/kg) exerted its protective effect against DEN-induced HCC probably by preventing membrane damage and loss of integrity, as well as by repairing hepatic tissue damage caused by carcinogenesis.

SOD is said to act as the first line of defence against superoxide radicals generated as a by-product of oxidative phosphorylation.[37] Further, GPx converts H_2O_2 to H_2O. GPx is capable of reducing free hydrogen peroxide and lipid peroxides. The level of GPx is used to monitor the balance of oxidative stress and chemopreventive ability. Decreased activity of SOD and GPx in DEN-treated mice could have been due to the overutilization of this nonenzymatic and enzymatic antioxidant to scavenge the products of lipid peroxidation. Our results indicated that DEN injection increased the susceptibility of hepatocytes to carcinogenesis thereby reducing the activity of SOD and GPx. However, ZPDC glycoprotein significantly increased the activity of SOD and GPx in liver tissue and serum (Figure 2). These results indicated that ZPDC glycoprotein might have had a protective effect against DEN-induced hepatocarcinogenesis.

It has been reported that HSPs are molecular chaperones that play crucial roles in protein folding and translocation. They are also implicated in cancer development.[38] It is known that levels of HSPs are elevated in many cancers and elevated HSP expression provides cellular resistance to anticancer therapies.[39] HSPs have been classified in at least six major families according to their approximate molecular size, namely HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs including HSP27.[7] Of these aforementioned sizes, HSP70 has been thought to be important in hepatitis c virus-related HCC as a potential molecular target in HCC treatment.[40] It has also been demonstrated that HSP70 could be a sensitive marker for the differential diagnosis of early HCC from preneoplastic lesions or noncancerous liver tissue.[41] In this study, we evaluated the expression of HSP70 levels using the Western blot method (Figure 3). A significantly elevated level of hepatic HSP70 resulted after treatment with DEN, however, it decreased after ZPDC glycoprotein treatment. The results suggested that ZPDC glycoprotein may have had a potent inhibitory effect against hepatocarcinogenesis.

NF-κB dysregulation may lead to more aggressive tumour behaviour in human hepatocellular carcinomas.[9] It has been reported that deleted NF-κB (p50) has an inhibitory effect on hepatocarcinogenesis in DEN-treated mice.[42] Also, it has been demonstrated previously that DEN-induced inflammation triggers ROS production, which activates NF-κB followed by release of pro-inflammatory cytokines, chemokines and COX-2, all of which events lead to hepatocarcinogenesis.[43] Generally, NF-κB consists primarily of two proteins (p50 and p65), which are referred to as RelA and c-Rel family. Under basal conditions, it is retained in the cytoplasm bound to an inhibitory subunit IκB. During a response to stimulators such as carcinogen, the p65 subunit dissociates from the IκB subunit and translocates from the cytoplasm to the nucleus, where it dimerizes with the p50 subunit and interacts with specific target genes, such as COX-2 leading to increased carcinogenesis processes.[44,45] Therefore, the result in this study examined the expression of NF-κB in nuclear protein extract. As shown in Figure 4, ZPDC glycoprotein had the ability to decrease the expression of NF-κB (p50 and p65) in liver treated with DEN.

MMPs (MMP-2 and MMP-9) have been suggested to be important in cancer cell invasion and angiogenesis because tumour cells must cross type IV collagen-rich basement membrane of vessel walls to spread to sites distant from the primary tumour.[46] MMP-2 is constitutively expressed by normal cells. However MMP-9 is the major enzyme that is considered to be an important contributor to the process of metastasis in various tumours.[47] Therefore, we examined the inhibitory effect of ZPDC glycoprotein on the expression of MMP-9 in DEN-treated ICR mice. As shown in Figure 5 DEN treatment significantly increased expression of MMP-9 compared with the control. Treatment with ZPDC glycoprotein significantly decreased the expression of MMP-9, compared with expression after DEN treatment alone.

COX-2 is chronically over-expressed in many premalignant, malignant and metastatic cancers, including HCC.[48] At least two isoforms of COX (COX-1 and COX-2) that have approximately 60% similarity in amino acids have been identified.[49] COX-1 is constitutively expressed with homeostatic roles in various tissues. On the other hand, COX-2 is an inducible isoenzyme which has been found to be locally induced by proinflammatory mitogens, cytokines and growth factors during inflammation and carcinogenesis. Recently, many research papers have indicated that COX-2 expression is used as a marker to detect the initiation of hepatocarcinogenesis.[50] It has been reported that its expression is considered to promote the release of active MMP-9. This study has shown that COX-2 expression induced by DEN decreased after ZPDC glycoprotein treatment (Figure 6). Thus, ZPDC glycoprotein might be able to prevent the MMP-9 expression throughout COX-2 signalling.

Conclusions

The results obtained from this study indicated that ZPDC glycoprotein (20 mg/kg) decreased the levels of LDH, ALT and TBARS but increased SOD and GPx activity in DEN-treated ICR mice. With respect to the hepatocarcinogenic indicator and hepatocarcinogenic signals, the activity of HSP70, NF-κB, COX-2, and MMP-9 decreased. The findings speculate that ZPDC glycoprotein prevented liver tissue from damage caused by DEN in the experimental mouse model. However, the precise mechanism by which ZPDC glycoprotein inhibits gene expression in hepatocarcinogenesis remains to be elucidated.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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