Antifibrotic activity of anthocyanidin delphinidin in carbon tetrachloride-induced hepatotoxicity in mice

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A B S T R A C T

The aim of this study was to investigate the hepatoprotective effects of anthocyanidin delphinidin in carbon tetrachloride (CCl4)-induced liver fibrosis in mice. Male Balb/C mice were treated with CCl4 dissolved in olive oil (20%, v/v, 2 mL/kg) intraperitoneally (i.p.), twice a week for 7 weeks. Delphinidin was administered i.p. once daily for next 2 weeks, in doses of 10 and 25 mg/kg of body weight. The CCl4 control group has been observed for spontaneous reversion of fibrosis. CCl4-administration induced an elevation in serum transaminase and alkaline phosphatase levels and increased oxidative stress in the liver. Delphinidin has successfully attenuated oxidative stress, increased matrix metalloproteinase-9 and metallothionein I/II expression and restored hepatic architecture. Furthermore, the overexpression of tumor necrosis factor-α and transforming growth factor-β has been withdrawn by delphinidin. Concomitantly, the expression of α-smooth muscle actin indicated returning of hepatic stellate cells (HSC) into inactive state. Our results suggest the therapeutic effects of delphinidin in CCl4-induced liver fibrosis by promoting extracellular matrix degradation, HSC inactivation and down-regulation of fibrogenic stimuli, with strong enhancement of hepatic regenerative capability.

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1. Introduction

Liver fibrosis is a frequent event which follows chronic damage to the liver, characterized by excess deposition of connective tissue in extracellular matrix (ECM) and a distortion of the liver tissue architecture by forming a fibrous scar (Friedman, 2000). Reactive oxidant species are directly involved in both onset and progression of fibrosis (Poli, 2000). Chronic carbon tetrachloride (CCl4) intoxication is a well-known model for producing oxidative stress and hepatic injury. Its biotransformation produces hepatic toxic metabolites, the highly reactive trichloromethyl free radical, which could be further converted to peroxynitrilochromethyl radicals and induce oxidative damage in the cell (Williams and Burk, 1990).

Anti-oxidants seems to be effective in the treatment of chronic liver damage and fibrosis (Parola and Robino, 2001). Polyphenols, naturally occurring anti-oxidants in fruits, vegetables and plant-derived beverages such as tea and wine, have been associated with a variety of beneficial properties (Havsteen, 2002). Among polyphenols, the anthocyanidins and their glycosylated derivatives, anthocyanins, abundantly present in pigmented fruits and vegetables, have attracted the attention of scientists over the last decade as they appear to possess potent activity against various pathological conditions. Anthocyanidin delphinidin has been shown to possesses anti-oxidant, anti-inflammatory, anti-mutagenic and anti-angiogenic properties (Noda et al., 2002; Hou et al., 2005; Azevedo et al., 2007). It exerts its action through distinct mechanisms, including vascular endothelial growth factor receptor-2 phosphorylation inhibition, epidermal growth receptor and platelet-derived growth factor ligand/receptor signaling, and cancer cell proliferation and invasion via modulation of Met receptor phosphorylation (Lamy et al., 2006, 2008; Afaq et al., 2008; Syed et al., 2008).

Activated hepatic stellate cells (HSC) play a major role in hepatic fibrogenesis and tissue regeneration (Friedman, 2000). The key factors involved in the activation of HSC could be divided into mitogenic cytokines (which stimulate HSC proliferation) such as transforming growth factor-α (TGF-α), platelet-derived growth factor (PDGF), interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and insulin-like growth factors (IGFs), and fibrogenic cytokines (which induce synthesis of matrix protein) like transforming growth factor-β1 (TGF-β1) and interleukin-6 (IL-6) (Tsukamoto, 1999). Upon liver injury, HSC became activated, converting themselves into a myofibroblast-like cells and producing extracellular matrix (ECM). ECM is regulated by a balance of synthesis and enzymatic degradation of ECM proteins (Arthur, 1994). The key enzymes responsible for the degradation of protein components of ECM and basement membrane are matrix metalloproteinases (MMPs). The...
activity of these enzymes is altered during the processes of fibrogenesis and fibrolysis (Knittel et al., 2000).

The metallothioneins (MTs), small cysteine-rich heavy metal-binding proteins, participate in an array of protective stress responses (Andrews, 2000). The induction of MT synthesis can not only protect animals from hepatotoxicity induced by various toxins including CCl4, but also play a role in repair and regeneration of injured liver (Cherian and Kang, 2006). Previously, we have shown that hepatoprotective activity of luteolin, another dietary anti-oxidant, is associated with increased MTs expression in both acute and chronic liver damage (Domitrović et al., 2008, 2009).

Because the specific treatments to stop progressive fibrosis of the liver are not available, the objective of the present study was to investigate the therapeutic effect and mechanisms of action of anthocyanidin delphinidin in chemically-induced liver fibrosis in mice.

2. Materials and methods

2.1. Materials

Delphinidin was purchased from Polyphenols Laboratories AS, Sandnes, Norway. Carbon tetrachloride (CCl4) was obtained from Kemika, Zagreb, Croatia. Olive oil and gelatin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of the highest grade commercially available.

2.2. Animals

Male Balb/C mice from our breeding colony, 2–3 months old, were divided into 5 groups with 5 animals per group. Mice were fed a standard rodent diet (pellet, type 4RF21 GLP, Mucedola, Italy) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% ash and 2.6 % lipids. Total energy of the diet was 16.4 MJ/kg. The animals were maintained at 12 h light/dark cycle, at constant temperature (20 ± 1 °C) and humidity (50 ± 5%). All experimental procedures were performed in compliance with the appropriate laws and institutional guidelines, and were approved by the Ethical Committee of the Medical Faculty, University of Rijeka.

2.3. Experimental design

CCl4 dissolved in olive oil (20%; 2 mL/kg body weight) was administered intraperitoneally (i.p.) twice a week for 7 weeks, except the control group which received vehicle only. Seventy-two hours after the last CCl4 injection, the CCl4 group was killed. The CCl4 control group was observed for spontaneous resolution of hepatic fibrosis for next 2 weeks. Delphinidin dissolved in saline was administered i.p. at a dose of 10 and 25 mg/kg daily for 2 weeks. Each value represents the mean ± SD for five mice.

2.4. Hepatotoxicity

Serum levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and cholinesterase (CHE), as markers of hepatocyte integrity and liver function, were measured by using a Bio-Tek EL808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) according to manufacturer’s instructions.

2.5. Determination of oxidative stress

Glutathione peroxidase (GSHPx) activity and ratio reduced/oxidized glutathione (GSH/GSSG) were measured spectrophotometrically, using Glutathione Peroxidase Assay Kit and Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), according to manufacturer’s instructions. Protein content in supernatants was estimated by Bradford’s method, with bovine serum albumin used as a standard (Bradford, 1976).

2.6. Determination of hepatic hydroxyproline

The tissue samples (50–60 mg) were hydrolyzed in 6 M HCl at 110 °C for 24 h. After being filtered through a 0.45 pm filter, samples were extracted and analyzed according to the procedure of Bergman and Loxley (1963) as described previously (Domitrović et al., 2009). Briefly, sample neutralization was obtained using 10 M NaOH and 3 M HCl. Chloramine T in citrate-acetate-buffer was added to the hydrolysates, followed by the addition of Ehrlich reagent. Tubes were incubated in a water-bath at 60 °C, and the absorbance of each sample was measured spectrophotometrically at 550 nm (Cary 100, Varian, Mulgrave, Australia).

2.7. Histopathology

Liver specimens were fixed in 4% phosphate buffered formalin, embedded in paraffin and cut into 4 μm thick sections. Sections for histopathological examination were stained with hematoxylin and eosin (H&E) and with Mallory trichrome stain for detection of collagen fibers. Hepatic fibrosis was assessed by use of the Ishak fibrosis score (Ishak et al., 1995) by a single pathologist blinded to the laboratory data. Stage of fibrosis was assessed using minimal fibrosis (Ishak score = 1), mild fibrosis (Ishak score = 2), moderate fibrosis (Ishak score = 3 or 4), severe fibrosis and incomplete cirrhosis (Ishak score = 5), cirrhosis (Ishak score = 6).

2.8. Immunohistochemistry

Immunohistochemical studies were performed on paraffin embedded liver tissues using mouse monoclonal antibodies against TNF-α diluted 1:50 (clone 52B83, Abcam, Cambridge, UK), TGF-β1 diluted 1:50 (Clone 2Ar2, Abcam, Cambridge, UK), MT I + II diluted 1:50 (clone E9; DakoCytomation, Carpinteria, CA, USA), and α-SMA diluted 1:100 (SPM332, Abcam, Cambridge, UK), employing DAKO EnVision® System (DAKO Corporation, Carpinteria, CA, USA) as described previously (Domitrović et al., 2009). Briefly, slides were incubated with peroxidase block, washed, and monoclonal antibodies supplemented with bovine serum albumin were added and incubated overnight at 4 °C in a humid environment. Finally, samples were incubated with peroxidase labeled polymer conjugated to secondary antibodies containing carrier protein linked to Fe fragments to prevent non-specific binding. The immunoreaction product was visualized by adding substrate-chromogen diaminobenzidine (DAB) solution. Tissues were counterstained with hematoxylin, dehydrated in gradient of alcohol and mounted with mounting medium. The intensity of staining was graded as weak, moderate and intense. The specificity of the reaction was confirmed by substitution of primary antibodies with irrelevant immunoglobulins of matched isotype, used in the same conditions and dilutions as primary antibodies. Stained slides were analyzed by light microscopy (Olympus BX51, Tokyo, Japan).

2.9. MMP zymography

The activity of MMPs was analyzed by gelatin zymography assay as described previously (Domitrović et al., 2009). After tissue homogenization in RIPA buffer

Table 1

<table>
<thead>
<tr>
<th>Serum markers of liver damage</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
<th>CHE (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.1 ± 2.5</td>
<td>11.6 ± 1.0</td>
<td>75.8 ± 8.4</td>
<td>3905 ± 112</td>
</tr>
<tr>
<td>CCl4</td>
<td>121.2 ± 9.8a</td>
<td>40.2 ± 2.5a</td>
<td>153.8 ± 10.4a</td>
<td>3320 ± 141a</td>
</tr>
<tr>
<td>CCl4 control</td>
<td>64.4 ± 7.8b</td>
<td>21.2 ± 3.7b</td>
<td>116.1 ± 17.5b</td>
<td>3668 ± 69b</td>
</tr>
<tr>
<td>CCl4 + delphinidin 10 mg/kg</td>
<td>41.2 ± 3.6ab</td>
<td>17.5 ± 1.5ab</td>
<td>94.7 ± 11.3ab</td>
<td>3950 ± 209ab</td>
</tr>
<tr>
<td>CCl4 + delphinidin 25 mg/kg</td>
<td>28.4 ± 2.3b</td>
<td>11.3 ± 0.4b</td>
<td>74.1 ± 6.5b</td>
<td>4140 ± 242b</td>
</tr>
</tbody>
</table>

Mice were given CCl4 dissolved in olive oil (20%; v/v, 2 mL/kg) intraperitoneally (i.p.) twice a week for 7 weeks, except the control group which received vehicle. Seventy-two hours after the last CCl4 injection, the CCl4 group was killed. The CCl4 control group was observed for spontaneous resolution of hepatic fibrosis for next 2 weeks. Delphinidin was administered i.p. at a dose of 10 and 25 mg/kg daily for 2 weeks. Each value represents the mean ± SD for five mice.

a p < 0.05 as compared with the control group.
b p < 0.05 as compared with the CCl4 control group.
Table 2
Relative liver weight, hydroxyproline content (Hyp) and anti-oxidant status in mice liver.

<table>
<thead>
<tr>
<th></th>
<th>Liver weight (g/100 b.w.)</th>
<th>Hyp (µg/g liver)</th>
<th>GSHPx (U/g protein)</th>
<th>GSH/GSSG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.01 ± 0.13</td>
<td>212 ± 19</td>
<td>60.8 ± 2.4</td>
<td>10.4 ± 0.9</td>
</tr>
<tr>
<td>CCl4</td>
<td>7.07 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>619 ± 79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.5 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCl4 control</td>
<td>6.51 ± 0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>769 ± 65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.2 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCl4 + delphinidin 10 mg/kg</td>
<td>6.29 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>385 ± 36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.6 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.9 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCl4 + delphinidin 25 mg/kg</td>
<td>5.82 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>255 ± 37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.3 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.3 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mice were given CCl4 dissolved in olive oil (20%, v/v, 2 mL/kg) intraperitoneally (i.p.), twice a week for 7 weeks, except the control group which received vehicle. Seventy-two hours after the last CCl4 injection, the CCl4 group was killed. The CCl4 control group was observed for spontaneous resolution of hepatic fibrosis for next 2 weeks. Delphinidin was administered i.p. at a dose of 10 and 25 mg/kg daily for 2 weeks. Each value represents the mean ± SD for five mice.

<sup>a</sup> p < 0.05 as compared with the control group.
<sup>b</sup> p < 0.05 as compared with the CCl4 control group.

10 µg of liver tissue protein lysates were separated by an 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 0.1% gelatin, at 4°C and 150 V for 5 h. Gels were washed for 30 min in 2.5% Triton X-100 to remove the SDS, and briefly washed in the reaction buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM CaCl2, 1 µM ZnCl2 and 0.02% NaN3. The reaction buffer was changed to a fresh one, and the gels were incubated at 37°C for 48 h. Gelatinolytic activity was visualized by staining the gels with 0.1% Coomassie blue R-350 for 4–5 min, destained with methanol–acetic acid–water (30:10:60, v/v/v) for 15–20 min. Dried gels were scanned (CanoScan LiDE 35, Cannon), and the intensity of the bands was assayed by computer image analysis software (NIH Image J software, available at http://rsb.info.nih.gov/ij).

3. Statistical analysis

Data were analyzed using StatSoft Statistica (Tulsa, OK, USA) version 7.1 software. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Dunnett’s post hoc test.

Fig. 1. Representative photomicrographs of liver sections stained with H&E. (A) Normal hepatic architecture in control mice. (B) Mice intoxicated with CCl4 for 7 weeks developed massive necrotic lesions (arrows) with microvesicular steatosis and mononuclear cell infiltration (arrowheads). (C) Two weeks later, ballooned hepatocytes with hyaline inclusions emerged (arrow). (D) Mice treated with 10 mg/kg of delphinidin showed only sporadic hepatic lesions (arrow). (E) Restored liver architecture in mice receiving 25 mg/kg of delphinidin. Original magnification 100×, insets 400×.
Fig. 2. The histopathological detection of collagen in the livers by Mallory trichrome staining. (A) Control mice showed traces of collagen only in vascular walls (arrow). (B) Mice receiving CCl\textsubscript{4} for 7 weeks developed fibrosis with occasional portal to portal bridging (arrows). (C) Two weeks later, abundant collagen deposits were present (arrows). (D) The collagen deposits in mice receiving 10 mg/kg delphinidin were significantly reduced (arrow). (E) Mice receiving 25 mg/kg of delphinidin showed no signs of parenchymal collagen. Original magnification 100×, insets 400×. (F) Scoring on fibrosis is represented as mean ± SD (n = 5). delph 10-delphinidin 10 mg/kg, delph 25-delphinidin 25 mg/kg.* p < 0.05 as compared with the control group and # p < 0.05 as compared with the CCl\textsubscript{4} control group.

test. Values in the text are means ± standard deviation (SD). Differences with p < 0.05 were considered to be statistically significant.

4. Results

4.1. Hepatotoxicity

Serum ALT, AST and ALP activities were significantly increased, whereas ChE activity was lower in mice receiving CCl\textsubscript{4} twice a week for 7 weeks (Table 1). In the CCl\textsubscript{4} control group, observed for the spontaneous regression of fibrosis for 2 weeks, the activity of transaminases and ALP decreased, and the ChE activity was improved when compared to the CCl\textsubscript{4} group. Delphinidin administration further attenuated changes in the level of serum markers of hepatic damage in a dose-dependent manner, returning their values to control levels in mice treated with delphinidin 25 mg/kg.

4.2. Oxidative stress and hydroxyproline content

The liver weight of CCl\textsubscript{4}-intoxicated mice markedly increased when compared to the control group, which was accompanied with increase in hydroxyproline content (Table 2). The GSHPx activity and GSH/GSSG ratio significantly decreased, suggesting development of oxidative stress. In mice observed for a spontaneous recovery from fibrosis, liver weight was similar to the CCl\textsubscript{4} group, however hydroxyproline content was even more increased. Additionally, oxidative stress was somewhat attenuated. Treatment with delphinidin further decreased the weight of livers and returned hydroxyproline content to values similar to controls in mice receiving 25 mg/kg of delphinidin, as well as it diminished oxidative stress.

4.3. Histopathology

Liver sections from control mice stained with H&E showed normal hepatic architecture. The livers of CCl\textsubscript{4}-intoxicated mice
The livers from control mice stained with Mallory trichrome stain showed normal hepatic architecture, with collagen fibrils visible only in the wall of adjacent blood vessels (Fig. 2A). Liver section from mice receiving CCl₄ for 7 weeks, sacrificed 72 h after the last CCl₄ injection, showed fibrotic scars within hepatic lesions, predominantly in the periportal areas, with early fibrotic septa developed as a thin fibrotic strands (Fig. 2B). In the CCl₄ control group, observed for spontaneous regression of fibrosis for additional 2 weeks, a markedly increased collagen bands intersected the liver parenchyma and formed bridging fibrotic septa, which progressed into pseudolobules (Fig. 2C). In mice treated with 10 mg/kg of delphinidin collagen deposits were significantly reduced in extent and were less frequent, compared to the CCl₄ control group (Fig. 2D). The livers of mice receiving 25 mg/kg delphinidin showed maintained histoarchitecture, similar to controls, with only sporadic pericellular fibrosis (Fig. 2E). Fig. 2F presents the Ishak fibrosis score for all experimental groups. Advanced hepatic fibrosis, characterized by long collagenous septa connecting the central veins across the liver parenchyma in the CCl₄ control group, was abrogated by the administration of delphinidin.

4.4. Immunohistochemistry

We performed immunohistochemical staining using chronic liver injury markers. The expression of α-SMA is a characteristic feature of activated HSCs and this is considered as a marker for hepatic fibrosis. In the livers of control animals α-SMA immunopositivity was restricted to the wall of majority of portal and central veins and arterial tunica media, while other hepatic cells remain negative (Fig. 3A). CCl₄-administration has induced strong perisinusoidal α-SMA expression in activated HSC, connected among themselves with thin, bridging immunopositivity (Fig. 3B). Activated HSC could be distinguished from other myofibroblasts of the...
Fig. 4. The expression and specific tissue distribution of TNF-α. (A) Liver sections from control mice. (B) Mice receiving CCl4 for 7 weeks showed TNF-α immunopositivity in macrophages/Kupffer cells lying within sinusoids (arrows). (C) Two weeks later, TNF-α immunoreactivity was even more pronounced (arrows). (D) Mice treated with low dose of delphinidin had decreased TNF-α immunopositivity within hepatic sinusoids (arrow). (E) In the group treated with high dose of delphinidin the immunoreaction was negative. Original magnification 400×. Representative immunohistochemical stain for TNF-α from five mice.

Liver by their specific position in the liver parenchyma (Cassiman et al., 2002). In the CCl4 control group, observed for spontaneous regression of fibrosis for additional 2 weeks, perisinusoidal immunostaining was significantly reduced (Fig. 3C). Mice receiving 10 mg/kg of delphinidin showed only sporadic α-SMA immunopositivity (Fig. 3D), while the livers of mice treated with 25 mg/kg of delphinidin showed staining pattern similar to control animals (Fig. 3E).

The livers of control mice did not show TNF-α immunoreactivity (Fig. 4A). In the CCl4 and CCl4 control group, TNF-α immunopositivity was observed mainly in necrotic areas of the liver affected with scar tissue formation (Fig. 4B and C), in the cells that according to morphology and localization correspond to the Kupffer cells. These cells tend to form clusters around damaged areas, which could represent the development of granuloma. In the livers of mice treated with low dose of delphinidin, weak TNF-α immunoreactivity could be observed in sinusoidal space (Fig. 4D). Hepatic TNF-α immunopositivity has been completely withdrawn by high dose of delphinidin (Fig. 4E).

Immunohistochemistry for triggering hepatic fibrosis was performed in the mouse livers using anti-TGF-β1 antibodies. In normal livers, both parenchymal and non-parenchymal cells were TGF-β1-negative (Fig. 5A). CCl4-intoxication has resulted in strong TGF-β1 immunoreactivity in non-parenchymal cells within the fibrotic septa (Fig. 5B), which was sustained in the CCl4 control group (Fig. 5C), correlating with enhanced deposition of collagen into ECM as observed by Mallory staining and hydroxyproline content. Macrophages strongly expressing TGF-β1 were infiltrated at the chronic injured area constituted with fibrotic septa around the central vein. Both low and high dose of delphinidin significantly reduced hepatic TGF-β1 expression (Fig. 5D and E, respectively), which was accompanied with the subsequent reduction in collagen deposits and the regression of liver fibrosis.

Fig. 6 shows the extent of hepatic MT I/II expression in experimental groups. In the control group, MT I/II immunopositivity was negligible (Fig. 6A). In the group treated with CCl4 for 7 weeks, sacrificed 72 h after the last CCl4 injection, week MT I/II immunopositivity was adjacent to non-parenchymal cells in areas affected by a massive necrosis (Fig. 6B). These cells morphologically correspond to Kupffer cells, which occasionally produce MTs (Leyshon-Sørland and Stang, 1993). In the CCl4 control group, observed for spontaneous regression of fibrosis for additional 2
Fig. 5. The expression and specific tissue distribution of TGF-β1. (A) Liver sections from control mice. Strongly positive macrophages are detected within the fibrotic septa in mice receiving CCl4 for 7 weeks (B) and 2 weeks later, in the CCl4 control group (C) (arrows). (D) Low dose of delphinidin significantly decreased TGF-β1 immunopositivity (arrows). (E) Mice treated with high dose of delphinidin had staining pattern similar to controls. Original magnification 400×. Representative immunohistochemical stain for TGF-β1 from five mice.

weeks, MT I/II immunopositivity was also found to be sporadic and restricted to the fibrous lesions, while in the remnant liver tissue was not detected (Fig. 6C). Similarly, in the livers of mice treated with delphinidin 10 mg/kg weak non-parenchymal MT I/II immunopositivity was present (Fig. 6D). On the other hand, the livers of mice treated with delphinidin 25 mg/kg showed intense MT I/II immunopositivity in hepatocytes, both cytosolic and nuclear (Fig. 6E).

4.5. MMP zymography

To investigate whether pro-MMP-9 or other pro-MMPs were activated during liver fibrosis and regeneration, protein extracts of liver tissues were subjected to zymographic analysis using gelatin as a substrate to detect any gelatinase activities. A representative result of 5 zymography experiments is shown in Fig. 7. Liver extracts in equal protein quantities (10 mg/kg) of hepatic homogenates contained mainly the latent form of MMP-9 at about 92 kDa (Fig. 7A). CCl4-intoxication slightly but significantly increased MMP-9 expression, which was sustained in the CCl4 control group 2 weeks later (Fig. 7B). Treatment with delphinidin resulted in an overexpression of MMP-9 in a dose-dependent manner, being the most prominent in mice treated with 25 mg/kg of delphinidin. The MMP-2 expression was below detection limit in all groups.

5. Discussion

Independently from the nature of the injury, the usual initiating event of fibrogenesis is represented by tissue necrosis of variable entity, followed by activation of large amount of fibroblasts and fibroblast-like cells for excess production of ECM (Tsukamoto, 1999). HSC play a major role in hepatic fibrosis (Friedman, 2000). Upon liver injury, HSC became activated, proliferating and converting themselves into a myofibroblast-like cells which produce large amount of ECM. HSC have been found to migrate in vitro (Marra et al., 1999), suggesting their ability to infiltrate into the fibrotic lesions and participate in the repair of damaged tissue (Kinnman et al., 2000). In this study, mice exposed to CCl4 showed necrotic changes resulting in the release of hepatic enzymes (AST, ALT, ALP) and impaired hepatic synthetic function marked by the decrease in serum ChE activity. Furthermore, increased perisinusoidal α-SMA
immunopositivity throughout affected lobuli in CCl₄-intoxicated mice suggested the activation of HSC and their transformation into myofibroblast-like cells, which was accompanied with increased collagen deposition. Hepatic collagen was even more abundant in the CCl₄ control group, suggesting development of progressive fibrosis, despite the reduced α-SMA immunostaining in this group. Withdrawal of α-SMA immunopositivity in the group treated with high dose of delphinidin indicates the regression of HSC into a quiescent state, which is critical for resolution of fibrosis (Iredale, 2001). The resolution of fibrosis was accompanied with normalization of serum AST, ALT, ALP and ChE levels, indicating the recovery of hepatic integrity and function.

Withdrawal of ECM produced by HSC is accomplished by a family of MMPs, however, the mechanisms of removing of fibrotic scars and re-expression of down-regulated MMPs during the resolution process are still unknown (Han, 2006). MMP-9, the 92 kD collagenase IV, is a major MMP in basement membrane-like ECM remodeling, secreted by neutrophil granulocytes, macrophages and leukocytes (Salguero et al., 2008), with HSC as an additional source (Knittel et al., 1999). MMP activity in the progression of liver fibrosis is regulated through expression of tissue inhibitor of metalloproteinases (TIMPs), which simultaneously prevent degradation of ECM (Iredale, 2001). During recovery from fibrosis TIMP level decreases while collagenase activity and the apoptosis of HSCs increase, which results in an increased fibrinolysis (Woessner, 1991). Delphinidin therapy has activated the expression of MMP-9, with the highest increase in the group treated with high dose of delphinidin. Our results are in agreement with previous findings, confirming the essential role of MMPs in removal of fibrotic scars.

Persistent and marked oxidative stress condition could be observed in many chronic pathological processes affecting hepatic tissue (Poli, 2000). Involvement of reactive oxygen species can be clearly demonstrated in fundamental events of hepatic fibrogenesis, like activation of HSC, expression of MMPs and their specific inhibitors. Oxidative stress can trigger a cellular stress response characterized by induction of anti-oxidants, acute phase reactants and heat shock proteins, which play a role in limiting tissue damage. In this study, delphinidin significantly reduced oxidative stress in the liver of CCl₄-treated mice in dose-dependent manner, as suggested by the restoration of hepatic GSHPx activity and GSH/GSSG ratio. Hepatoprotective effects of delphinidin, at least in part, could originate from its free radical scavenging ability (De Lima et al., 2004).
cells in the area affected with fibrotic lesions. TNF-α expression is dependent upon TNF-β activity, compared to untreated groups. The bar graph shows the extent of MMP-9 expression (mean ± SD, n = 5). * p < 0.05 as compared with the control group and # p < 0.05 as compared with the CCl4 control group.

Fig. 7. A representative SDS-PAGE gelatin zymogram. MMP-9 was identified by molecular weight relative to markers. (A) Expression of latent form of 92 kDa MMP-9 in experimental groups. (B) Densitometric analysis of MMP-9 expression detected in gelatin zymograms. Delphinidin treatment significantly increased the MMP-9 activity, compared to untreated groups. The bar graph shows the extent of MMP-9 expression (mean ± SD, n = 5). * p < 0.05 as compared with the control group and # p < 0.05 as compared with the CCl4 control group.

2007). The reduction of oxidative stress is a hallmark of improvement in pathophysiologic conditions in the liver.

Cytokines TNF-α and TGF-β1 have a number of functions that could be important in liver injury and repair. In the liver, TGF-β is produced by Kupffer cells and HSC, exerting predominantly profibrogenic activity in injured liver tissue (Osawa et al., 2009). Transdifferentiation of HSC into myofibroblasts is associated with an increased expression and activation of TGF-β1. After hepatic injury, the newly recruited non-parenchymal cells produce significant levels of TGF-β, which ultimately lead to recruitment and proliferation of mesenchymal cells at the site of inflammation (Armendariz-Borunda et al., 1990). Further, TGF-β may function as a down-regulator of hepatocyte proliferation because hepatocyte DNA synthesis could be inhibited by TGF-β (Russell, 1988). Additionally, TGF-β1 inhibits production of the matrix-degrading enzymes, while simultaneously up-regulates expression of TIMPs (Overall et al., 1989). In this study, TGF-β1 expression in necrotic areas of the livers suggests that macrophages/Kupffer cells were actively producing this profibrotic cytokine, consequently activating HSC and increasing the production of ECM. Concomitantly with the resolution of fibrosis, TGF-β1 expression was diminished.

TNF-α is a pleotropic cytokine produced mainly by Kupffer cells, associated with a variety of physiological and pathophysiological conditions (Beyaert and Fiers, 1998). Hepatic expression of TNF-α has been found to rise in chronic liver damage of various etiology (Luster et al., 1999). Liver fibrosis resulting from chronic CCl4 exposure is dependent upon TNF-α, which could be accompanied with reductions in procollagen and TGF-β synthesis, suggesting that TNF-α is responsible for regulating products that induce inflammation and fibrosis in CCl4-induced hepatotoxicity (Simeonova et al., 2001). On the other hand, several studies demonstrated that TNF-α is involved in liver repair (Brucoleri et al., 1997; Yamada et al., 1997). In this study, moderate TNF-α immunopositivity in the CCl4 group suggests its active production by macrophages/Kupffer cells in the area affected with fibrotic lesions. TNF-α expression was stronger in the CCl4 control group, suggesting pronounced and sustained pro-inflammatory response. The therapeutic activity of delphinidin was associated with the decrease in TNF-α production, concomitantly with the histological improvement in the livers.

The induction of MT synthesis is known to protect animals from hepatotoxicity induced by various toxins including CCl4, but also play a role in repair and regeneration of injured liver (Cherian and Kang, 2006). Transcription of MT genes is rapidly and dramatically up-regulated in response to agents which cause oxidative stress and/or inflammation (Chubatsu and Meneghini, 1993; Andrews, 2000). Moreover, MTs have the essential role in cell proliferation and liver regeneration, with increased MT expression in both the cytoplasm and nucleus of rapidly proliferating cells (Apostolova and Cherian, 2000; Miles et al., 2000). MT may activate metalloenzymes and transcription factors associated with gene transcription, cell replication, differentiation and growth (Włostowski, 1993; Coyle et al., 2002; Jiang and Kang, 2004). The results of this study have shown low expression of MT I/II in all groups, except in mice treated with high dose of delphinidin. The liver sections from mice treated with delphinidin 25 mg/kg had markedly up-regulated MT I/II expression, both cytoplasmic and nuclear, which was accompanied with the resolution of fibrotic scar tissue, suggesting their direct involvement in the regeneration of injured liver tissue.

In conclusion, the delphinidin therapy has resulted in a complete reversal of progressive liver fibrosis by decreasing hepatic fibrogenic potential through inactivation of HSC and down-regulation of TNF-α and TGF-β expression, while stimulated MT I/II expression and enhanced hepatic regenerative capability. The results obtained from this study suggest the therapeutic application of delphinidin to promote liver regeneration in patients with hepatic fibrosis, although further studies are required.

Conflicts of interest

The authors declare that they do not have any conflict of interest.

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