Jerusalem artichoke attenuates experimental hepatic fibrosis via modulation of apoptotic signaling and fibrogenic activity

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ABSTRACT

Hepatic fibrosis is a central pathological process in a wide spectrum of liver diseases. This study aims to evaluate possible anti-fibrotic effect of Jerusalem artichoke tubers (JAT) and to investigate possible biochemical and molecular mechanisms for such effect. 60 male albino rats were divided equally (20 rats/group) into the following groups Group I: Untreated control group. Group II: Carbon tetrachloride (CCL4) treated group, rats given CCl4-olive oil (1:1, 2.8 ml/kg followed by 1.4 ml/kg after one week) by gavage. Group III: rats received JAT (1 g/kg body weight) orally starting from day one of CCL4 treatment. Biochemical analysis of liver enzymes activities and total bilirubin levels in serum besides histopathological evaluation, immuno-histochemical and western blotting analysis for P53, Bax and transforming growth factor-β (TGF-β) protein expression in liver tissue were executed. CCL4 treatment showed evident hepatocellular damage and fibrosis, major biochemical and histopathological changes and disrupted expression patterns of P53, Bax and TGF-β proteins in liver tissue. On the other hand, JAT ameliorated most of these changes and restored expression levels of these proteins. In conclusion, JAT treatment showed promising hepatoprotective effect against CCl4-induced fibrosis via modulation of apoptotic signaling and fibrogenic activity.

Keywords: Liver fibrosis, P53, Bax, TGF-β, Jerusalem artichoke tubers.

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INTRODUCTION

A variety of insults including viral hepatitis (especially hepatitis B and C), alcohol abuse, drugs, metabolic diseases due to overload of iron or copper, autoimmune attack on hepatocytes or congenital abnormalities can cause chronic hepatic injury leading to fibrosis (Abdel-Wahab et al., 1994; Frank et al., 2000). Egypt has the highest prevalence of adult hepatitis C virus (HCV) infection in the world, affecting an average of 15 to 25% of the population in rural communities. Worldwide, HCV is one of the major causes of chronic liver diseases, which include inflammation, fibrosis and cirrhosis. Furthermore, HCV has been associated with increased morbidity and mortality in hepatocellular carcinoma (Shaheen and Myers, 2007; Sebastiani, 2009; Lin et al., 2011).

Hepatic stellate cells (HSCs) are directly involved in mediating the pathological changes that lead to the development of liver fibrosis. Following fibrogenic stimuli, HSCs change from quiescent to activated cells, which include increased proliferation, a phenotypic transformation to myofibroblast-like cells, increased synthesis of extracellular matrix proteins and contractility (Bataller and Brenner, 2005). During hepatic fibrogenesis, HSCs are activated by inflammatory cytokines and growth factors in paracrine and autocrine manners. Among them, transforming growth factor-β1 (TGF-β1) is well documented to be the most important fibrogenic stimulator for HSCs (Friedman, 2003). Suppression of HSC activation (such as inhibition of
NFkB activation pathways) and proliferation have been proposed as therapeutic strategies for the treatment and prevention of the hepatic fibrosis (Lotersztajn et al., 2005).

Recently, it has been proposed that plant-derived antioxidants may emerge as potential antifibrotic agents (Gebhardt, 2002; Schuppan et al., 1999). For example, it has been reported that herbal extracts such as Shosai-to (TJ-9) (Shimizu et al., 1999), silymarin (Boigk et al., 1997) and Inchin-ko-to (TJ-135) (Sakaida et al., 2003) can inhibit HSC activation and thereby exert anti-fibrotic effects in animals.

In this study, we aim to evaluate the anti-fibrotic effect of JAT and to offer possible mechanisms for such effect. Considerable interest has been generated in JAT (Helianthus tuberosus L.). It was reported to have possible anti-diabetic, hepatoprotective and anti-oxidant potential but little is known about possible mechanisms by which it exerts these effects (Kim and Han, 2013). The root has negligible amounts of fat and contains zero cholesterol. Nevertheless, its high-quality phyto-nutrition profile comprises of dietary fiber (non-starch carbohydrates), and antioxidants, in addition to small proportions of minerals, and vitamins (Slavin, 1997). It is one of the finest sources of dietary fibers, especially high in oligo-fructose inulin, which is a soluble non-starch polysaccharide. Inulin is a zero calorie, saccharine, and inert carbohydrate, which does not metabolize inside the human body, and thereby; make this tuber an ideal anti-oxidant (Slavin, 1997). The tuber contains small amounts of anti-oxidant vitamins such as vitamin C, vitamin A, vitamin E. These vitamins together with flavonoid compound like carotenes help scavenge harmful free radicals, and thereby offer protection from inflammation and cancer (El-Houfi, 2005).

MATERIALS AND METHODS

Animals

Sixty male albino rats (150 to 200 g) were purchased from the animal house colony, National Research Centre, Cairo. They were housed in standard conditions and received a standard diet and water ad libitum during the entire period of experimentation. All animals received human care according to the National Institute of Health guidelines [USA].

Chemicals

CCl4 was obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAX, P53 and TGF-β primary antibodies and secondary antibody for immuno-histochemical studies and western blot analysis were purchased from (Santa Cruz Biotechnology, CA).

JAT preparation

JAT was washed and sliced individually to thickness of approximately 1 mm. The slices were then soaked in acidic solution (lemon juice combined with citric acid and ascorbic acid) to inhibit polyphenol oxidase activity. Thereafter, they were subjected to dryness process by solar energy, blended, packed and kept in deep freezer (Tehone et al., 2005).

Experimental design

Rats were divided equally into three groups (20 rats/group). Control rats were treated with olive oil (2.8 ml/kg followed by 1.4 ml/kg after one week). CCL4 treated group: hepatic injury was induced by treating rats by gavage with CCI4-olive oil (1:1. 2.8 ml/kg followed by 1.4 ml/kg after one week) (Salam et al., 2007). JAT + CCL4 treated group: starting on the time of the first dose of CCI4 administration, rats also received JAT (1 g/kg body weight) orally (Zaky, 2009). The animals were killed on day 15 after the first dose of CCI4 or olive oil administration. Rats had free access to food and drinking water during the study.

Sample preparations

At the end of the experiment the blood was withdrawn from the carotid vein by cutting with fine scissors. Blood was collected by allowing it to drip into non-EDTA containing test tube. Serum was separated by centrifugation for 15 min at 3000 rpm. Samples were stored in aliquots in eppendorf tubes and frozen at -80°C. Livers were quickly removed, individual liver weights were accurately recorded, divided into two portions, one for histological examination; the second was immediately frozen in liquid nitrogen, kept at -80°C for western blotting.

Biochemical analysis

Diagnostic kits for serum alanine aminotransferase (ALT) and aspartate amino transferase (AST) were purchased from (Biodiagnostic CO., Egypt). Diagnostic kits for bilirubin were purchased from Randox Laboratories Co., UK.

Histopathological investigation

Liver slices were fixed in 10% formaldehyde and embedded in paraffin wax blocks. Sections of 5 μm thick were stained with hematoxylin and eosin (H&E), and masson trichrom (MT) stain then examined under light microscope for determination of pathological changes. The histological evaluation of the hepatic injury was semi-quantitatively scored by two independent observers who undertook the evaluation using an ocular micrometer by light microscopy. The severity of the hepatic injury was scored viz. (-), no injury; (+), mild; (++) moderate, and (+++) severe. The examination of the hepatic injury consisted of the evaluation of the following: vena centrals, portal area, and sinusoidal congestion, ballooning degeneration of hepatocytes and its location, the presence of focal parenchymal necrosis, Polymorphonuclear leukocytes (PMNL) infiltration and mononuclear leukocytes (MNL) infiltration; karyolysis of hepatocyte nuclei, the presence of pyknosis, loss of intercellular borders and disintegration of hepatic cords.

Morphometric analysis of hepatic fibrosis score was performed on Hematoxylin and Eosin (H&E) and Masson's trichrome (MT) stained liver sections (X400) using semi-quantitative fibrosis scores. Two different blinded persons determined the fibrotic scores for 20 rats, which were used in mean for the analysis. Healthy liver was classified as 0. Fibrous expansion of the portal areas was scored as 1. Stage 2 denotes septal fibrosis with marked fibrous septa, and stage 3 was characterized by portal-portal septa (bridging fibrosis but intact architecture). The advanced fibrotic stage 4 (that is, cirrhosis, characterized by bridging fibrosis with nodules) (Bancroft and Gamble, 2002).
Table 1. Liver enzymes activities and total bilirubin levels in serum.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal control</th>
<th>CCL4</th>
<th>CCL4+JAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/L)</td>
<td>30.8 ± 2.898</td>
<td>141.17 ± 1.471***</td>
<td>91.71 ± 5.498 $$$</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>22.45 ± 2.26</td>
<td>92.5 ± 1.516 ***</td>
<td>75.21 ± 1.523 $$$</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.4436 ± 0.093</td>
<td>1.852 ± 0.483 ***</td>
<td>1.052 ± 0.0605 $$$</td>
</tr>
</tbody>
</table>

Data are expressed as X ± SD of 20 rats in each group (n = 20). Significant difference between groups was analyzed by t-student test, where: (P < 0.05 is considered significant) (* CCL4 was compared to normal control, * CCL4+ JAT group was compared to CCL4 group).

Immuno-histochemical analysis of BAX, P53 and TGF-β

Liver tissue sections were subjected to de-waxing, hydration and thermal induction antigen retrieval. Slices were blocked and incubated with anti-BAX antibody (1:200) anti-p53 antibody (1:100) and anti-TGF-β antibody (1:200) which were diluted in phosphate buffer saline solution 4°C overnight. The following day, the slices were washed and incubated with secondary antibodies. The slices were then incubated with diaminobenzidine tetrachloride for 5 to 10 min to develop the color, and staining was observed under microscope (Taylor, 1994).

Western blotting of BAX, P53 and TGF-β

Proteins were separated by 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% bovine serum albumin for 2 h, and then incubated overnight at 4°C with anti-BAX antibody (1:200) anti-p53 antibody (1:100) and anti-TGF-β antibody (1:200). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, diluted) at room temperature for 2 h, and then washed again and detected by the enhanced chemiluminescence reaction (Towbin et al., 1979). Actin acted as a loading control to ensure even loading and transfer. The intensities of the bands were analyzed by Image J software.

Statistical analysis

Data were estimated using analysis of variance and all values are expressed as mean ± SD. P value < 0.05 was considered significant. All analyses were implemented by SPSS 20 software for Windows (Chicago, IL, United States).

RESULTS

Effect of JAT on serum markers of hepatic injury induced by CCl4

ALT and AST activities and total bilirubin levels were significantly increased in the CCl4 treated group compared to control group (P < 0.001), which was significantly reduced by JAT treatment (P < 0.001) (Table 1).

JAT attenuates hepatic fibrosis induced by CCl4

After 8 weeks of CCl4 administration, liver histopathology was significantly changed. CCl4 treated group showed moderate to marked congestion in the central vein and severe congestion in the portal area and sinusoids Parenchymal necrosis was observed. PMNL infiltration was mild whereas mononuclear leukocytes MNL infiltration was mild in the central vein, severe in the portal area and moderate in the sinusoids. Moderate karyolysis and pyknosis, and severe loss of intracellular borders and disintegration of hepatic cords were observed and reflected by high hepatic fibrosis score (Figures 1 and 2, and Table 2). JAT treatment exhibited some degree of hepatoprotective potential against CCl4 as evident by the presence of normal hepatic cords, absence of necrosis and lesser fatty infiltration with subsequent decrease in hepatic fibrosis score (P < 0.001) (Figures 1 and 2, and Table 3).

Effect of JAT on liver protein expression of TGF-β, Bax and P53

Both TGF-β and Bax showed high cytoplasmic expression in CCL4 group. In contrast, JAT treatment showed reduction in the expression of both of them (Figures 3 and 4). In addition, p53 showed high nuclear expression with further cytoplasmic translocation. P53 immunopositive cells reached up to 70% of the stained area compared to 5% observed in the normal control group. On the other hand, in JAT treated group the expression level of p53 was markedly reduced (Figure 5).

Immuno-histochemical analysis was consistent with western blotting results which showed the same pattern. TGF-β, Bax and p53 proteins were overexpressed in the CCl4 group while clearly reduced in JAT treated group (Figure 6).

DISCUSSION

Till now few effective, safe and convenient approaches for liver fibrosis are clinically available. In this study we offer a possible natural hepato-protective agent which can effectively ameliorate CCL4- induced liver fibrosis.

In the present study, CCL4 treatment induced marked fibrosis and architectural distortion as stated by previous studies (Wang et al., 1997; Mehmetcik et al., 2008). It
also resulted in significant increase in serum ALT, AST activities as well as total bilirubin levels. These biochemical parameters have been reported to be sensitive indicators of liver injury and have been found to be elevated in CCL4-induced hepatotoxicity (Arıcı and Çetin, 2011; Kim and Han, 2013; Moreno-Otero and Trapero-Marugán, 2010). These enzymes are present in cytoplasmic area of the cell and are released into circulation due to hepatic cell membrane fluidity and permeability (Wang et al., 1997). JAT treatment significantly improved these biochemical changes and succeeded to attenuate histopathological manifestations of hepatic fibrosis and lowered fibrosis score (Salazar-Montes et al., 2000). It could be speculated that this hepatoprotective effect of JAE may be in part related to its antioxidant activity. Oxidative stress is reportedly associated with chronic liver diseases of various etiologies, and ROS may act as or upon signaling molecules in the pathways or networks of hepatic fibrosis (Friedman, 2003; Poli, 2000; Parola and Robino, 2001).

The effects of JAT on hypoglycemia and hepatoprotection in streptozotocin (STZ)-induced diabetic rats were also investigated. Oral administration of JAE may have a potential benefit of ameliorating diabetic symptoms by improving liver damage caused by STZ (Wang et al., 1997).

Injury of hepatocytes results in the recruitment and stimulation of inflammatory cells, as well as resident ones, including Kupffer cells. Factors released by these inflammatory cells lead to activation of HSCs and their transformation into a myofibroblast-like phenotype. Chronically activated HSCs produce large amounts of extracellular matrix proteins (ECM) and enhance fibrosis by secreting a broad spectrum of cytokines such as TGF-β1. This exerts pro-fibrotic actions in other cells and in an autocrine manner perpetuates their own activation (Guo et al., 2013).

Our result also showed marked increase in the expression of TGF-β in CCL4 treated group. TGF-β is an important cytokine in the regulation of ECM production. Strategies aiming at disrupting TGF-β synthesis and signaling cascade markedly suppress liver fibrosis.
development (Bataller and Brenner, 2005; Friedman, 2003; Lotersztajn et al., 2005). Events taking place in livers of cirrhotic rats indicate the presence of sustained injury in the liver maintaining a constant inflammatory process. JAT helped in reducing the expression levels of TGF-β may be via down-regulation of collagen promoter activity and antagonized TGF-β-stimulated collagen gene transcription (Bataller and Brenner, 2005; Friedman, 2003; Lotersztajn et al., 2005).

The regulation of apoptosis is a potential mechanism through which many agents may prevent hepatotoxicity and cancer. Noteworthy, consequences from the toxin-induced excessive oxidative stress, depletion of antioxidant enzymes and induction of membrane lipid peroxidation may prompt the extrinsic or intrinsic apoptotic pathways. These pathways eventually lead to the activation of apoptosis (Tsamandas et al., 2003).

We have found that the expression level of the expression of P53 and the pro-apoptotic protein, Bax, were increased in CCl4-induced hepatic injury. Guo et al. (2013) reported that Bax was over regulated in CCl4 induced hepatotoxicity (Inagaki et al., 2003). Tsamandas et al. (2003) also reported that western blot analysis showed an increased Bax expression toward advanced fibrotic stages. Increased Bax expression in fibrosis implies that they are responsible for hepatocyte depletion through apoptosis, during progress of liver fibrosis and fibrous tissue accumulation, until cirrhosis is established (Castilla et al., 1991). Our results demonstrated that JAT effectively reduced p53 and Bax expression levels. These anti-apoptotic effects were related to decreases in the expression of pro-apoptotic proteins in the cytoplasm and the inhibition of proteins associated with apoptosis in the mitochondria (Flier et al., 1993).

**Conclusion**

JAT treatment showed promising hepatoprotective effects, and this study is unique to demonstrate the hepatoprotective role of JAT. The results of the present study add further evidence that JAT can ameliorate CCl4-induced liver injury.

<table>
<thead>
<tr>
<th>Group</th>
<th>Congestion</th>
<th>PMNL</th>
<th>MNL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Central vein</td>
<td>Portal space</td>
<td>Sinusoid</td>
</tr>
<tr>
<td>Normal control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCL4</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CCL4+JAT</td>
<td>++</td>
<td>+++</td>
<td>++</td>
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</tbody>
</table>

**Table 2.** Histopathological evaluation for different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ballooning degeneration localization and Necrosis</th>
<th>Karyolysis and pyknosis</th>
<th>Loss of intercellular borders</th>
<th>Disintegration of hepatic cords of cords</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCL4</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CCL4+JAT</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
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</table>

The severity of the hepatic injury was scored viz. (-), no injury; (+), mild; (++), moderate, and (+++) severe. The examination of the hepatic injury consisted of the evaluation of the following: vena centralis, portal area, and sinusoidal congestion, ballooning degeneration of hepatocytes and its location, the presence of focal parenchymal necrosis, Polymorphonuclear leukocytes (PMNL) infiltration and mononuclear leukocytes (MNL) infiltration, karyolysis of hepatocyte nuclei, the presence of pyknosis, loss of intercellular borders and disintegration of hepatic cords.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Mean rank</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0.275 ± 0.002</td>
<td>-------</td>
</tr>
<tr>
<td>CCL4</td>
<td>2.233 ± 0.12***</td>
<td>0.0001</td>
</tr>
<tr>
<td>CCL4+JAT</td>
<td>1.164 ± 0.05 $$$</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

**Table 3.** Morphometric analysis of hepatic fibrosis.

Morphometric analysis of hepatic fibrosis score was performed on Hematoxylin and Eosin (H&E) and Masson's trichrome (MT) stained liver sections (X400) using semi-quantitative fibrosis scores. Two different blinded persons determined the fibrotic scores for 20 rats, which were used in mean ± SD for the analysis. Healthy liver was classified as 0. Fibrous expansion of the portal areas was scored as 1. Stage 2 denotes septal fibrosis with marked fibrous septa, and stage 3 was characterized by portal-portal septa (bridging fibrosis but intact architecture). The advanced fibrotic stage 4 (that is, cirrhosis, characterized by bridging fibrosis with nodules). Comparison of liver fibrosis score between different groups using Mann-Whitney U test. (P < 0.05 is considered significant) * CCL4 was compared to normal control, # CCL4+ JAT group was compared to CCL4 group.)
Protective effect against CCl4-induced fibrosis via modulation of apoptotic signaling and fibrogenic activity.
Figure 6. Western blotting for TGF-β, Bax, P53 and β-Actin protein expression in liver homogenate. CCL4 treated group (B) showed increased liver protein expression in all parameters compared to normal untreated group (A) which was reduced by JAT treatment (C). β-actin acted as loading control to ensure even loading.

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