Attenuation of CCl₄ Induced Oxidative Stress, Immunosuppressive, Hepatorenal Damage by Fucoidan in Rats

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Abstract

The protective and therapeutic effects of fucoidan extract from Laminaria species against liver damage induced by CCl₄ in rats was investigated by monitoring the serum level and hepatic m-RNA expression of TGFβ-1, liver and renal markers, as well as oxidative stress and antioxidant biomarker. Thirty six adult male albino rats were divided into 4 equal groups; one was used as a negative control while groups II, III, and IV administrated 0.1 mL/100 g body weight twice a week for 8 weeks with carbon tetrachloride (CCl₄), fucoidan (400 mg/kgbwt orally/day), and CCl₄ plus fucoidan, respectively. Blood samples were collected at the end of experiment and sera were separated to evaluate serum levels and the hepatic m-RNA expression of transforming growth factor beta (TGFβ-1), tumor necrosis factor (TNF α), interferon gamma (IFN-γ), interleukin (IL), IL-1β, IL-6 and IL-10, antioxidant markers, reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and lipid peroxidation malondialdehyde (MDA) as well as selective biochemical markers of liver and kidney functions were estimated. The results of this investigation revealed that treatment with fucoidan improved elevated expression of liver TGF β-1, II-1β, IL-6, TNF α and serum level of malonialdehyde (MDA), total bilirubin (T. Bil), induced by CCl₄ at 8th week post treatment. In addition to enhancing the antioxidant enzyme activities, GSH, GPx, CAT and SOD. Also, liver transaminase (ALT, AST), alkaline phosphatase (ALP), reduced in fucoidan and CCl₄ treated group. These results show that crude fucoidan has potential immunomodulatory, antioxidant and hepatoprotective effects against the hepatic damage induced by CCl₄.

Keywords: Fucoidan; CCl₄; Hepatoprotective; Oxidative stress; Cytokine; Rats

Introduction

Liver diseases are among some of the fatal diseases in the world today, they pose a serious challenge to international public health. Hepatic fibrosis is a wound healing response to chronic liver injury which is characterized by a net accumulation of extracellular matrix (ECM) including collagen, glycoproteins, and proteoglycan [1-3]. Hepaticstellate cells (HSCs), previously known as Ito cell that under physiological conditions stores 80% of retinoids (vitamin A), are the cytological base of hepatic fibrosis. The quiescent HSC is transformed with progressive injury into myofibroblast like cells that are characterized by the appearance of cytoskeleton protein α smooth muscle actin (α SMA) and collagen-1 considered as a biomarker for HSCs activation. TGFβ-1 is a key molecule and an important fibrogenic cytokine that facilitates the activation of HSCs and converts it from static HSCs to the phenotype of myofibroblast to express a SMA and possess the character of contraction [4-6].

Carbon tetrachloride, CCl₄ has been a frequently used chemical to experimentally induced hepatic fibrosis. Depending on the dose and duration, the effect of CCl₄ on hepatocytes is manifested histologically as hepatic statues, fibrosis, hepatocellular death and carcinogenic. The hepatotoxic effect of CCl₄ is attributed to its immediate cleavage by cytochrome P450 (CYP2E1) in hepatocytes, which generates trichloromethyl radicals leading to lipid peroxidation and subsequently to membrane damage. The activated Kupffer cell produces toxic metabolites (inflammatory cytokines and reactive oxygen intermediates which results in the injury of hepatic parenchymal cells [7-11].

Fucoidans, is a sulfated polysaccharide extracted from the cell wall of brown algae and some marine invertebrates. It contains substantial complement, antioxidant, and anti-inflammatory activities. In addition, it is used as immunomodulatory and blood lipid reducing agent, and has acted against hepatorenalpathy and possesses gastric protective effect. Moreover, Fucoidan extracted from the brown seaweed Laminaria japonica had a hepatoprotective effect [11-14].

The aim of the present study is to evaluate the hepatoprotective effect of fucoidan on liver fibrosis induced by CCl₄ in rats, through detection of gene expression and serum cytokines of TGFβ-1, IL-1β, IL-6, TNF α, IFN-γ and IL-10, in addition to oxidative stress reactions and biochemical hepatorenal markers.

Material and methods

Experimental animals

Thirty two, 1-2 month old male albino rats were involved in the present study. The rats were kept in galvanized zinc-plate cages under strict hygienic conditions and were ensured free from any infection.
The rats were maintained for one week on a pelleted diet and water ad libitum before starting the experiment for acclimatization. The experiment was approved according to the ethical committee of our college.

**Chemicals**

CCl₄ was purchased from Sigma Aldrich (Co, USA), Primer sequences for PCR amplification The primer of selected pro-inflammatory cytokines were obtained from (Thermo scientific Co. USA) as displayed in Table 1. Fucoidan extract of Laminaria species received as a powder from Sigma Aldrich was used as a freshly prepared solution dissolved in normal saline.

**Liver antioxidant analysis**

Liver specimens were rapidly detached, rinsed in ice cold saline buffer (20 mM Tris–HCl, 0.14 M NaCl buffer, pH 7.4) and homogenized in the saline buffer (10%, w/v). The homogenate aliquots were kept at -30 °C for MDA and antioxidant markers estimation. The oxidative stress marker, MDA and antioxidant system SOD, CAT, GPx, and GSH were determined enzyme linked immunoassay (ELISA), using ready-made kits (Cayman. Co. USA) according to the enclosed pamphlets.

**Statistical analysis**

Data were analyzed by means of one way ANOVA using the SPSS software statistical program with post-hoc LSD multiple comparison test using SPSS software (SPSS for Windows ver. 21.00, USA). Data are expressed as the mean ± SE, and P<0.05 was considered statistically significant.

**Results**

**Cytokines parameters**

The gene expression and serum cytokines TGF-β1, L-1β, TNFα and IL-6 were significantly higher in the CCl₄-treated group at 8th week post treatment as compared with the control rats (Table 2 and Figure 1). On the other hand, no significant changes were observed in the aforementioned cytokine expression and serum levels in fucoidan treated groups when compared with the control group.

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**Serum biochemical analysis**

Ready frozen serum samples were analyzed for ALT, AST, gamma glutamyl-transferase (GGT), ALP, total bilirubin, direct bilirubin, glucose, total protein, albumin, urea, creatinine were determined with a semi-automatic spectrophotometer (BM-Germany 5010) using commercial test kits (Randox Co. UK) according to stander laboratory method.

**RT-PCR analysis**

Expressions of mRNAs for the proinflammatory cytokines, TGF-β1, IL-1β, IL-6, TNFα, IL-10 and TNF-α, were quantified by real-time RT-PCR. Total RNA was isolated from liver specimens using the RNA Easy kit (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany), according to the stander technique. The extracted RNA was dissolved in 30 μL nuclease-free distilled water and stored at -30°C until used. The concentration and purity of RNA were determined by Nanodrop Spectrophotometer (Thermo Scientific, USA). Preparation of the RNA / primer mixture was achieved by adding an RNA template. Real-time PCR was performed using 2 μL templates in a 20-μL reaction containing 0.25 μM of each primer and 12.5 μL Sybr Green. The mixture was incubated at 70-75°C for 5-10 min and then placed at room temperature for 5-10 min for denaturation and primer annealing. The RT-PCR mixture was prepared and completed by adding 10 μL of RNA/primer mixture. The thermal profiles that were used consisted of denaturation at 95°C, for 15 s, 60°C for 20 s, and 72°C for 60 s followed by 45 cycles of 95°C for 15s, and a final elongation at 72°C, in a real-time PCR machine (Applied Bio-system Thermo Fisher, USA). The quantitative mRNA expression level of targeted pro-inflammatory cytokines were estimated by determining the cycle threshold (CT), which is the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence. The reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as a control. The selective cytokine gene expression was calculated using the 2-CT according to Livak and Schmittgen (2001).

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| Primer Name | Primer Sequence (5′–3′) | Base pairs |
|--------------|------------------------|------------|
| TGF-β-FW     | TAT AGC AAC AAT TCC TGG CG | 162        |
| TGF-β-RW     | TGC TGT CAC AGG AGC AGT G | 73         |
| IL-1β-FW     | CAC CTT CTT TTC CAT CTT TG | 32         |
| IL-1β-RW     | GTC GTT GCT TGT CTC TCC TTG TA | 84       |
| IL-6-FW      | TGA TGG ATG CTT CCA AAC TG | 75         |
| IL-6-RW      | GAG CAT TGG AAG TGG GGG TA | 162        |
| TNF-α-FW     | ACT GAA CTT CGG GGT GAT TG | 73         |
| TNF-α-RW     | GCT TGG TGG TTT GCT ACG AC | 32         |
| IFN-γ        | TGG CAT AGA TGT GGA AGA AAA - | 75         |
IFN-γ-RW | TGC AGG ATT TTC ATG TCA CCA -  
IL-10-FW | TGC CTT CAG TCA AGT GAA GAC  
IL-10-RW | AAA CTC ATT CAT GGC CTT GTA  

Table 1: Primers used for Real-time PCR Amplification.

Figure 1: Hepatic mRNA expression of transforming growth factor beta (TGFβ-1), tumor necrosis factor (TNF α), interferon gamma (IFN-γ.), interleukin (IL), IL-1 β, IL-6 and IL-10, in the experimental groups.

Additionally, TGF-β1, L-1β, TNF α and IL-6 expression and serum levels were lower in the fucoidan and CCl₄ treated group than CCl₄ treated group. Also, IFN-γ, was reduced in fucoidan and CCl₄ treated group as compared with CCl₄ group, while IL-10 was non-significant differ between all treated groups, as shown in Table 2.

Table 2: Liver Expression and Serum Cytokines Markers (Mean ± S.E) at 8th week Post Treatment with CCl₄, and Fucoidan.

| Parameters (Pg/mL) | Experiment Groups |
|-------------------|-------------------|
|                   | Control | CCl₄ | F | F+CCl₄ |
| TGF-β1            | 14.1 ± 3.42 | 35.2 ± 2.01 | 13.2 ± 1.26 | 19.5 ± 1.18 |
| IL-1β             | 38.7 ± 2.52 | 59.5 ± 3.62 | 38.7 ± 2.52 | 49.9 ± 3.82 |
| TNF-α             | 21.5 ± 2.41 | 65.2 ± 1.71 | 22.4 ± 0.45 | 36.6 ± 0.48 |
| IL-6              | 19.4 ± 1.84 | 35.3 ± 0.62 | 18.5 ± 0.78 | 20.3 ± 0.64 |
| IFN-γ (Pg/mL)     | 36.8 ± 2.82 | 22.4 ± 1.86 | 38.12 ± 3.99 | 34.9 ± 3.49 |
| IL-10             | 12.2 ± 0.32 | 8.1 ± 0.62 | 13.1 ± 1.58 | 10.1 ± 0.32 |

Table 3: Hepatic Antioxidant and Oxidative Stress Biomarkers (Mean ± S.E) at 8th week post Treatment with CCl₄, and Fucoidan.

| Parameters    | Experiment Groups |
|---------------|-------------------|
|               | Control | CCl₄ | F | F+CCl₄ |
| MDA (µM)      | 65.1 ± 3.09 | 67.4 ± 4.01 | 68.25 ± 4.36 | 70.5 ± 5.08 |
| GSH (µM)      | 71.5 ± 3.54 | 38.8 ± 3.51 | 82.25 ± 3.66 | 66.6 ± 4.96 |
| GPx (µM)      | 9.5 ± 0.94 | 6.45 ± 0.82 | 0.45 ± 0.98 | 8.15 ± 1.54 |
| CAT (µM)      | 3.5 ± 0.62 | 1.88 ± 0.08 | 6.65 ± 0.68 | 3.01 ± 0.23 |
| SOD (µM)      | 221.9 ± 5.10 | 195.2 ± 4.06 | 216.2 ± 3.09 | 200.8 ± 2.06 |

Biochemical Parameters

Results presented in Table 4 show a significant increase in the ALT, AST, ALP and GGT serum activities and total bilirubin, as well as urea and creatinin, while there was a significant decrease in albumin and glucose and a non-significant change in total protein in CCl₄-treated group when compared with the control group. All of the aforementioned biochemical markers, did not significantly change in the fucoidan treated group alone, as compared with the control group. Furthermore, the hepatic markers only were improved in fucoidan and CCl₄ treated group when compared with CCl₄ treated group alone, as displayed in Table 4.

Table 4: Hepatorenal Biomarker Profiles (Mean ± S.E) at 8th week Post Treatment with CCl₄, and Fucoidan.

| Parameters    | Experiment Groups |
|---------------|-------------------|
|               | Control | CCl₄ | F | F+CCl₄ |
| ALT (U/L)     | 20.5 ± 1.24 | 34.1 ± 1.23 | 19.5 ± 1.81 | 30.3 ± 2.19 |
| AST (U/L)     | 28.1 ± 1.42 | 38.9 ± 1.26 | 29.2 ± 1.34 | 35.3 ± 2.14 |
| GGT (U/L)     | 18.5 ± 1.05 | 65.2 ± 4.25 | 17.6 ± 1.95 | 41.3 ± 3.18 |
| ALP (U/L)     | 10.9 ± 0.54 | 18.4 ± 0.50 | 9.8 ± 0.41 | 16.2 ± 0.42 |
| T. Bili. (mg/dl) | 0.48 ± 0.03 | 0.71 ± 0.04 | 0.52 ± 0.05 | 0.65 ± 0.06 |
| Dir. Bili (mg/dl) | 0.22 ± 0.02 | 0.46 ± 0.09 | 0.24 ± 0.04 | 0.42 ± 0.06 |
| Glucose (mg/dl) | 112.8 ± 2.20 | 87.2 ± 4.53 | 109.6 ± 2.52 | 86.6 ± 4.54 |
| T. Protein (gm/dl) | 3.25 ± 0.18 | 2.98 ± 0.22 | 3.16 ± 0.25 | 3.25 ± 0.21 |
| Urea (mg/dl)  | 55.8 ± 1.28 | 69.5 ± 1.45 | 54.2 ± 1.02 | 66.6 ± 3.23 |
| Creatinine (mg/dl) | 0.51 ± 0.01 | 0.69 ± 0.02 | 0.54 ± 0.04 | 0.68 ± 0.03 |

Antioxidant and lipid peroxidation parameters

Results obtained showed a significant decrease (P<0.05) in antioxidant markers, GSH, CAT, SOD and GPx in the CCl₄-treated group when compared with the control rats. In addition, lipid peroxidation MDA was significantly higher in CCl₄-treated group when compared with the other experimental group. On the other hand, treatment with fucoidan alone caused a significant increase in GSH, and CAT as compared with the control group. The antioxidant markers, GSH, CAT, GPx and lipid peroxidation (MDA), did not significantly differ in fucoidan and CCl₄ treated group from those of the control group as displayed in Table 3.

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Discussion

The liver plays a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. Importantly, it is the main detoxifying organ of the body, which removes wastes and xenobiotics by metabolic conversion and biliary excretion [13].

CCl₄ metabolism is an established model of liver necrosis and fibrosis. The liver damage caused by this metabolism is free radical dependent as CCl₄ is oxidized by cytochrome P450 to the highly reactive trichloromethyl (CCl₃) radicals that are generated by the reductive cleavage of CCl₄ bond and generated oxygen radicals and phospholipid peroxides. These generated trichloromethyl free radicals cause liver necrosis, destruction of ECM and lipid peroxidation of membranes. Results from this investigation revealed that TGF-β1 mRNA expression increased as fibrosis developed in CCl₄ induced liver fibrosis in treated rats. As TGF-β1 activity is enhanced by proteolytic release and activation of latent TGF-β1 from HSC. Other several studies have confirmed that the stimulation and proliferation of HSCs are the crucial points in the production of ECM, resulted in the differential of HSCs into myofibroblasts with production of α-SMA lead to stable liver fibrosis. Moreover, the results show that serum TGF-β1, TNF, and IL-6 were significantly increased in CCl₄ treated group as compared with the control. Tan et al. recorded significant elevation of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β with hepatic fibrosis in mice treated with CCl₄. In the same line, Ahn et al. observed elevated pro-inflammatory cytokines, including TNF-α and IL-1β, mRNA expression with hepatic damage in rats treated with CCl₄. At the molecular level, CCl₄ activates TNF-α, TGF-β1, and IL-6 production that appear to direct the cell toward destruction or fibrosis, while IL-10 counteract the liver fibrogenesis. In this context, Fue et al. and Tan et al. concluded the elevation the inflammatory cytokines have a key role in pathogenesis of liver fibrosis and activation of HSCs [14-22].

Fucoidan, a family of sulphated polyolucose polysaccharides, exhibit a variety of biological properties, anti-inflammatory, antibacterial, immunostimulant and antioxidant. The biological effects of fucoidan relate to their polysaccharide backbone and sulfate content. Recently, the anti-fibrotic activity of fucoidan was reported in an animal model of hepatic fibrosis. The serum's TGF-β1, TNF-α and IL-6, in addition to mRNA liver expression, were reduced in rats treated with fucoidan and CCl₄ at 8th week post treatment when compared with CCl₄ rats. This is in agreement with results obtained by Shini and colleagues who discovered that fucoidan treatment attenuates HSCs activation by inhibiting TGF-β1. Also, Jinging et al. concluded the fucoidan down regulation of TGF-β1 and reduce the HSCs activation and the formation of ECM. In addition, researchers reported that elevation of reactive oxygen species, is the key to HSC activation and release of inflammatory cytokines. In the same aspect, Park et al. concluded that fucoidan applies anti-inflammatory effects by inhibiting the expression of pro-inflammatory cytokines in vitro and in vivo, together with a restricted antibacterial effect in vivo. Furthermore, fucoidan enhanced the production of pro-inflammatory cytokines, IL-6, IL-8 and TNF-α in human neutrophil and delay neutrophil apoptosis [17-26].

Malondialdehyde is a reactive aldehyde, used as an indicator of the amount of lipid peroxidation. This can be ascribed to the polyunsaturated fatty acids’ damage caused by ROS; this damage results in different products, including MDA. In the present study, there was a significant increase in serum MDA concentration in the CCl₄ treated group; this agrees with the findings reported by other researchers. Lipid peroxidation (LPO), is one of the principal causes of CCl₄ induced liver and renal injury. Attack by free radical oxygen species (ROS) on the polyunsaturated fatty acids generates different products, including aldehydic products, resulting eventually in a loss in the membrane’s integrity. Antioxidant enzymes such as SOD, CAT, GPx and GSH constitute a helpful team of defense against ROS, hydroperoxide and environmental toxicity. Likewise, glutathione is a first line of defense and scavenges ROS. Additionally, GSH-dependent enzymes offer an important line of protection as they detoxify noxious byproducts generated by ROS. The depletion concentration of GSH in the liver may be due to enhanced GSH utilization in the elimination of peroxides or NADPH reduction activity. Several studies showed that GSH plays a key role in detoxifying the toxic metabolites of CCl₄ and that liver injury begins when GSH stores are markedly depleted. Tan et al. and Ahn et al. observed a significant reduction of the antioxidant system, GSH, CAT, GPx and GR, while marked elevation of lipid peroxidation, MDA in mice and rats treated with CCl₄ respectively. In the present study, marked reduction in the antioxidant system (SOD, CAT, GPx and GSH) in CCl₄ treated groups was observed when compared with the control group. Depletion of the antioxidant system in CCl₄ treated group could be attributed to CCl₄ generated cellular ROS production and the subsequent depletion of the antioxidant cellular system [27-35].

In fucoidan treated group, GSH and CAT were higher than that of the control group; this is due to the antioxidant activities of fucoidan which have been documented by Wang et al. Moreover, fucoidan reduced the lipid peroxidation, MDA elevation in CCl₄ treated groups. This is in line with results obtained by other researchers who found that I/P administration of fucoidan extract resulted in reduced high MDA level induced by CCl4 treatment in rats. On the other hand, Lie et al. reported that fucoidan from L. japonica had no effect on lipid peroxidation induced by FeSO₄ in vitro, and Nakazato et al. have indicated that the crude fucoidan extract did not reduce the high MDA level in liver injury induced by N-nitroso-diethylamine. Our results, however, found an elevation in GSH, CAT and GPx in rats treated with fucoidan and CCl₄ as compared with CCl₄ group. The increase in these enzyme activities was probably a response towards the increase in ROS generation since fucoidan has strong scavenging free radical activity, especially against superoxide radicals. This is in agreement with the findings of Jing et al. who reported that fucoidan exhibit radical scavenging activity, in vitro and antioxidative activity against oxidative stress in cellular model. Moreover, fucoidan has been reported to have a great potential in preventing free radical synthesis that mediates diseases and can prevent the increase of lipid peroxide in the serum, liver and spleen of rats and mice (Lie; Omar et al.). Furthermore, Phull et al. demonstrated the fucoidan is a potent antioxidant that can effectively reapel oxidative stress and arthritis-mediated inflammation. In addition to, the fucoidan inhibit expression of nitric oxide (NO), and exhibited antioxidant activity by reducing the reactive oxygen species (ROS) in microglia cells (Nguyen et al.). In the same line, Subash et al. recorded the levels of oxidative stress markers SOD, GPx, GSH, were reduced in inflammatory hepatocytes of rats treated orally with dexamethasone and fucoidan (300 mg/kg) [33-40].

CCl₄ administration causes severe liver damage demonstrated by a significant elevation of serum AST and ALT levels till the end of the experiment. This elevation may be attributed to the cellular leakage and damage of structural integrity of the liver cells. Similarly, CCl₄,
treatment induced elevation of serum GGT and ALP with high level of total bilirubin, and direct bilirubin, which are considered indicators of cholestasis and pathological alterations of the biliary flow. The highest concentration of direct bilirubin in the serum is an indication of liver injury caused by CCl4. Similar to results, other research groups reported elevation of liver marker enzymes and bilirubin in rats intoxicated with CCl4. Additionally, nephrotoxicity of CCl4 in the present study was manifested by elevation of urea and creatinine serum levels at 8th week post treatment (Table 3) in CCl4 treated groups, as compared with the control group. This is similar to results obtained by others. In the present study, administration of CCl4 to normal rats induced hepatic and renal toxicity, as CCl4 mediated peroxidation of lipid structures, enhances reactive oxygen species (ROS) and depletion of protein content of tissues; this results in sub cellular damage. Total blood protein level insignificantly changed in CCl4 treatment, while albumin was lower than the control group (Table 3). CCl4 intoxication leads to hypomethylation of cellular components; in the case of RNA the outcome is thought to be inhibition of protein synthesis. Hypoproteinemia and hypoalbuminemia in rats intoxicated with CCl4 for 6 weeks have been reported by Al-Yahya et al. In the present study, serum glucose was reduced in CCl4 a treated animal as hepatic glycogen content was decreased, reflecting decreased gluconeogenesis by the liver. Similar results were obtained by Rui et al., who reported that gluconeogenesis and Krebs cycle fluxes are altered in rat livers following CCl4 intoxication. The elevation of hepatic biochemical marker enzymes (ALT, AST, ALP, GGT) was reduced in fucoidan and CCl4 treated groups revealed improve liver function. The hepatoprotective of the fucoidan against CCl4 toxicity could be due to down regulation of inflammatory mediators and antioxidant activity of the fucoidan [33–43].

Finally, we concluded that crude fucoidan inhibit TGF-β, suppresses hepatic inflammation and attenuates hepatic oxidative stress in rats intoxicated with CCl4. Fucoidan could be a promising potential agent as a hepatoprotective and treatment of hepatic fibrosis.

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