Modulatory Effect of Wheat Germ Oil on Intestinal Oxidative Stress and DNA damage Induced by Carbon tetrachloride in Mice

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ABSTRACT

Background/Aim: The liver is continuously linked to the gut via the portal vein supply. Gastrointestinal complications are directly associated with liver cirrhosis. Carbon tetrachloride (CCl₄) caused liver toxicity is well documented in animal models, the very fewer search has been carried out on the intestinal damage in case of acute liver injury. Thus, this study aimed to investigate the intestinal alteration and subsequently the potential therapeutic role of Wheat Germ Oil (WGO) in the liver and small intestine after acute administration of CCl₄.

Material and Methods: Mice were randomly divided into 4 groups; control group, WGO group: received corn oil orally for 2 days then WGO (1400mg/kg) orally for 8 days, CCl₄ group: received CCl₄ orally for 2 days then corn oil for 8 days, CCl₄ + WGO group: received CCl₄ for 2 days then WGO for 8 days. Serum lipid profile, serum lactate dehydrogenase (LDH), intestinal oxidative stress enzymes, histopathological and DNA fragmentation assays were estimated.

Results: Acute dose (50%, 1 mL/kg body weight) of CCl₄-induced hyperlipidemia, hypocholesteremia, elevation in LDH, malondialdehyde (MDA), nitric oxide (NO) and intestinal DNA damage in addition to the reduction in the intestinal oxidative stress markers and an alteration in the mucosal architecture. On the contrary, WGO administration has the potency to protect not only the liver but also the small intestine in acute CCl₄-induced tissue damage. The valuable effect is chiefly attributed to its mechanism of reducing the lipid profile and suppressing the oxidative stress that caused DNA damage.

Conclusion: WGO administration could markedly improve the liver and the small intestine from the CCl₄ damage and consequently may be used as a therapeutic agent against the hepatic and intestinal toxicity.

INTRODUCTION

Carbon tetrachloride (CCl₄) is commonly used for experimental induction of liver injury in rodents to mimic the oxidative stress situations in many pathophysiological conditions (Zhu et al., 2013; Go et al., 2016). The single acute dose of CCl₄ causes liver toxicity that is characterized by hepatocellular necrosis and steatosis while a chronic dose of CCl₄ causes liver cirrhosis. Generation of free radicals has been observed in many tissues; liver, kidney, intestine, heart, lung, brain, and blood after CCl₄ administration (Dashti et al., 1989). The free radicals generated from CCl₄ and its metabolites induced an impairment of the endoplasmic reticulum (ER) and altered the permeability of the mitochondrial membrane, which leads to accumulation of lipids, reduction of protein synthesis and overproduction of the oxidative stress (Weber et al., 2003). Oxidative stress exhibits a significant role in the pathogenesis of liver injury and other hepatic alterations through implications between free radicals generated by CCl₄ and lipid peroxidation (Gutiérrez et al., 2010). The toxicity of CCl₄ is originated through its breakdown to the highly reactive trichloromethyl radical (CCl₃) in the endoplasmic reticulum by cytochrome P₄₅₀ enzymes. CCl₃ which rapidly reacts with oxygen forming trichloromethyl peroxy radical (CCl₃OO), that interacts with lipids causing lipid peroxidation that leads to hepatotoxicity (Risal et al., 2012). The free radicals produced from CCl₄ not only affect the cellular permeability of the hepatocytes leading to the elevation of hepatic enzymes but also cause deterioration in hepatic function through an elevation in lipid profile such as total lipids, triglycerides, cholesterol and HDL-cholesterol (Essawy et al., 2012).
The gut and the liver are bidirectionally communicated together through digestion and absorption products, bile, hormones and inflammatory mediators (Bajaj et al., 2012). The correlation between liver damage and the functional integrity of the intestine was documented by (Manevska 1975). As long as the gastrointestinal tract is affected, the mucosal alterations may arise, resulting in portal hypertension during liver cirrhosis (Llovet et al., 1994). Oxygen free radicals generated from CCl₄ play an important role in the damage of gut epithelial cells, which may affect the gut barrier function, facilitate bacterial translocation and induce the release of endotoxin (Ramachandran et al., 2002). Oxidative stress in the intestinal mucosa caused an elevation in xanthine oxidase which is an important source of free radicals in the small intestine (Watkins 2001).

Moreover, numerous herbal antioxidants could protect organs against CCl₄ induced oxidative stress by reducing lipid peroxidation and restoring the antioxidant enzymes activities. The essential oils including WGO have attracted much attention in recent years due to their unique nutritional value (Majzboob et al., 2016). WGO has a high amount of policosanol contents specially octacosanol, which reduces the cholesterol levels and improves the lipid profile (Anwar et al., 2015). WGO plays an important role in decreasing lipid peroxidation by activating the tocopherol redox-system (Leenhardt et al., 2008), as an interesting source of natural antioxidant tocopherols, sterols, and vitamin B complex. In addition, WGO has a high vitamin E and phenolic contents which act as inhibitors of the oxidation processes in body tissues and protect cells against the damaging effects of free radicals generated from CCl₄ (Liu et al., 2015).

Moreover, WGO not only prevents autodestruction of unsaturated fatty acids but also, confers DNA protective effects (Gelmez et al., 2009). Although the effect of WGO on improving liver toxicity is well documented. However, very few research has been carried out on the intestinal alterations in the case of acute liver injury.

Thus, the aim of this study was carried out to investigate the effect of acute oral dose of CCl₄ not only on the liver injury but also on the redox balance of the intestinal mucosa by assessing different levels of the oxidative stress, histopathological changes, and the intestinal DNA damage. It also aims to evaluate the potential role of WGO in preventing the deleterious effects of the CCl₄-induced acute liver injury in mice.

MATERIALS AND METHODS

Animals and chemicals
Male C57 BL/6 mice (6 weeks old, weighing 22-25 g) were obtained from National Research Center (NRC, Giza, Egypt). Mice were randomly grouped and housed in a conventional clean facility. All the experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals. Mice were given food and water ad libitum and maintained in a friendly environment with a 12 h/12 h light-dark cycle at room temperature (22°C–25°C). Mice were adapted to laboratory conditions for 7 days before beginning of the experiment. CCl₄, WGO, and all other chemicals were purchased from Sigma-Aldrich Biotechnology (St Louis, MO, USA). Assays kits for the detection of liver lipid profile and oxidative stress markers were purchased from Biodiagnostic (Giza, Egypt).

Induction of liver injury and WGO administration
Induction of liver injury in mice was done by oral administration of CCl₄ (50%, 1 mL/kg body weight) dissolved in corn oil, the dose was chosen to induce liver injury as previously reported (Srivastava et al., 2010). WGO was given to the animals by oral gavage at dose (1400 mg/kg body weight), according to previous literature (Karabacak et al., 2011).

Experimental design
Mice were randomly divided into 4 groups (6 mice/group):

- **Group 1:** Control group: mice received 1 mL oral administration of corn oil for 10 days.
- **Group 2:** WGO group, mice received corn oil orally for 2 days then WGO (1400mg/kg b.w) for 8 days.
- **Group 3:** CCl₄ group, mice received CCl₄ dissolved in corn oil orally for 2 days then received corn oil for 8 days.
- **Group 4:** CCl₄ + WGO group, mice received CCl₄ dissolved in corn oil orally for 2 days then received WGO for 8 days.

Animal handling
At the end of the 10th day, all the mice were euthanized after anesthesia; blood samples were collected and centrifuged at 2000 g for 20 min. Serum was stored at −20°C until used for biochemical assays. The small intestine was removed and immediately divided into two parts: the first part was stored at −80°C for biochemical studies and DNA fragmentation while the second part was suspended in 10% formal saline for fixation preparatory to histological processing.

Small intestine homogenate preparation
Small intestines were homogenized (10 % w/v) in icecold 0.1M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 2000 g for 15 min at 4°C and the resultant supernatant was used for the biochemical analyses.

Biochemical analysis of serum lipid profile
The appropriate kits (Bio-Diagnostic, Dokki, Giza, Egypt) were used for the determination of total lipids (Zöllner et al., 1962), triglycerides (Stein et al., 1960), serum total cholesterol and high-density lipoprotein -cholesterol (Tietz et al., 1995) according to the manufacturer’s instructions. Then, the concentration of LDL-cholesterol was calculated according to Friedewald’s formula (Friedewald et al., 1972). LDL-cholesterol
DNA was reprecipitated finally with absolute alcohol and estimated colorimetrically with the Griess reagent, which is a specific reaction for nitric oxide (NO).

**Lactate dehydrogenase (LDH)**

LDH activity as a marker of tissue injury was measured in the serum spectrophotometrically using commercially available kits (Saluca, Haansberg, Netherlands). LDH activity and expressed by U/L ultraviolet kit (Saluca, Haansberg, Netherlands) (Van der Heiden et al., 1994).

**Oxidative stress assessment**

**Malondialdehyde (MDA) assay**

The tissue content of MDA, an index of oxidative stress-mediated tissue lipid peroxidation, was determined by the thiobarbituric acid (TBA) method, as described previously (Ohkawa et al., 1979). The small intestine was homogenized with ice-cold 0.1M Tris-HCl buffer (pH 7.4) and the values of MDA in tissue homogenate were expressed as nmol/g tissue.

**Reduced glutathione (GSH) and Glutathione-S-transferase (GST) of the small intestinal tissue**

GSH and GST play a major role in protecting cells against damage from reactive oxygen species (ROS). Tissue GSH (mg/g tissue) and GST (nmol/min/g tissue) were assessed in the homogenate according to the manufacturer’s instructions (Habig et al., 1974; Beutler et al., 1963) respectively.

**Superoxide dismutase (SOD) assay**

The method of measuring the SOD activity depends on the inhibition of nitro blue tetrazolium (NBT) reduction by xanthine-xanthine oxidase after addition of superoxide dismutase (Nishikimi et al., 1972). SOD activity was expressed as unit/g of the intestinal tissue.

**Catalase (CAT) assay**

The catalase activity in the intestinal tissue was determined by the method described by Aebi (1984), in which the supernatant was incubated with H2O2 for one minute, and then the reaction was stopped with catalase inhibitor. The color intensity of the complex formed by chromophore and H2O2 was inversely proportional to the amount of catalase in the sample and measured at 510 nm. CAT activity was expressed as unit/g of the intestinal tissue (Aebi 1984).

**Nitric Oxide (NO)’ assay**

Nitric oxide (NO) is a free radical, an uncharged molecule with an unpaired electron. The reaction of NO with oxygen or other free radicals generates reactive nitrogen species (RNS), which cause multiple biological effects. Nitrite was estimated colorimetrically with the Griess reagent (Montgomery et al., 1961). The content of nitric oxide in the tissue was measured at 540 nm and expressed as umol/L.

**Histopathological preparation**

The small intestines were fixed in 10% neutral buffered formalin solution and embedded in paraffin wax blocks. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) then examined under light microscope for determination of pathological changes (Meyer 1903).

**DNA fragmentation assay**

Apoptosis is characterized by DNA fragments that are produced as a result of the endonucleolytic attack. According to Sambrook’s protocol (Sambrook et al., 1989), the intestinal tissues were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA and 0.2% triton-100x) for 20 min on ice before centrifugation, and digested with proteinase-K at 50°C (50 mg/ml) for 2 h. After digestion, DNA was extracted three times with phenol-chloroform and precipitated finally with absolute alcohol and 2.5 M sodium acetate (pH 4.0), then centrifuged at 5000 g for 30 min. DNA was washed three times with 70% ethanol dissolved in TE buffer (pH 8.0) and digested for 15 min at 37°C with DNase-free RNase-A (1 μg/ml). DNA was re-extracted in chloroform and precipitated with absolute ethanol and 2.5 M sodium acetate (pH 4.0).

A specific quantity (15 mg/lane) was loaded onto an agarose gel. DNA ladder was determined by a constant voltage (60 V) on a 1.2% agarose gel containing 0.4 mg/ml ethidium bromide. The gel was illuminated and photographed under UV light to record DNA fragmentation.

**Statistical analysis**

Data are expressed as mean ± SE of six mice in each group. Treated groups were compared with control and CCl4 group by using Unpaired Student’s t-test *P < 0.05, **P < 0.01 vs control group, #P < 0.05, ##P < 0.01 vs CCl4. SPSS, for Windows (USA, version 19.0) was used for the statistical analysis.

**RESULTS**

**Serum lipid profile**

Data recorded in Table 1 reports a significant increase (P < 0.01) in the serum levels of total lipids, triglycerides, total cholesterol, LDL-cholesterol and VDL-cholesterol in CCl4 group as compared to the corresponding control group. However, CCl4 treatment significantly decreased (P < 0.01) serum HDL-cholesterol in comparison to control group. On the other hand, oral administration of WGO for CCl4 treatment group resulted in a significant decrease (P < 0.01) of total lipids, triglycerides, total cholesterol, LDL-cholesterol and VDL-cholesterol levels and significant increase of HDL-cholesterol as compared to the corresponding CCl4 treatment group.
Lactate dehydrogenase (LDH)

To evaluate the cellular injury, LDH was measured spectrophotometrically in the serum. Serum LDH was found to be elevated significantly ($P \lessdot 0.01$) after CCl$_4$ treatment as compared to the control group, whereas WGO administration resulted in a significant attenuation of LDH level ($P \lessdot 0.01$) (Figure 1).

Intestinal oxidative stress

The current study revealed that MDA and NO levels were increased significantly ($P \lessdot 0.01$) in CCl$_4$-treated mice as compared to the control group. While, a significant decrease ($P < 0.05$) in the levels of GSH, GST, SOD and CAT were observed in the CCl$_4$-treated group. In contrast, the levels of MDA and NO were decreased significantly in CCl$_4$ + WGO group when compared to the CCl$_4$-treated group.

On the other hand, a significant increase in concentrations of GSH and CAT ($P < 0.05$), and GST, SOD ($P < 0.01$) were observed subsequent to the oral administration of WGO after CCl$_4$ treatment (Figure 2).

Table 1: Effect of WGO on serum lipid profile in mice treated with CCl$_4$

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Lipids (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Total cholesterol (mg/dL)</th>
<th>HDL-cholesterol (mg/dL)</th>
<th>LDL-cholesterol (mg/dL)</th>
<th>VLDL-cholesterol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>591.90 ± 56.26</td>
<td>113.91 ± 1.63</td>
<td>120.52 ± 2.99</td>
<td>69.26 ± 0.72</td>
<td>32.64 ± 2.68</td>
<td>21.16 ± 0.53</td>
</tr>
<tr>
<td>WGO</td>
<td>425.43 ± 40.54</td>
<td>111.7 ± 2.36</td>
<td>111.7 ± 3.33</td>
<td>72.86 ± 2.15</td>
<td>25.66 ± 1.01</td>
<td>23.14 ± 0.25</td>
</tr>
<tr>
<td>CCl$_4$</td>
<td>1108 ± 56.26*</td>
<td>153.70 ± 2.35*</td>
<td>166.20 ± 9.42*</td>
<td>33.90 ± 1.26*</td>
<td>94.23 ± 3.332*</td>
<td>30.73 ± 0.44*</td>
</tr>
<tr>
<td>CCl$_4$+WGO</td>
<td>468.5 ± 39.27***</td>
<td>115.63 ± 5.05***</td>
<td>128.80 ± 3.06***</td>
<td>61.33 ± 1.14***</td>
<td>44.35 ± 2.810**</td>
<td>23.13 ± 1.01***</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE; (n = 6). Significant difference is indicated by *$P < 0.05$ & **$P < 0.01$ vs control group and #*$P < 0.05$ & ##*$P < 0.01$ vs CCl$_4$.

Fig. 1: Effect of WGO on CCl$_4$-induced elevation in serum Lactate Dehydrogenase (LDH) levels as an index of general tissue damage.

Fig. 2: Effect of WGO on CCl$_4$-induced changes in antioxidant markers of the small intestine.

MDA = Malondialdehyde; GSH = Glutathione Reduced; GST = Glutathione-S-transferase; SOD = superoxide dismutase; CAT = Catalase; NO = Nitric Oxide.

Data are expressed as mean ± SE; (n = 6). Significant difference is indicated by *$P < 0.05$ & **$P < 0.01$ vs control group and #*$P < 0.05$ & ##*$P < 0.01$ vs CCl$_4$. 
Histopathological examination
Sections of the small intestine for control and WGO groups showed the normal architecture of healthy tissues including the intact mucosa, cylindrical glandular epithelium and the finger-like villi structure (Figure 3 A, B).

Acute toxicity of CCl₄ has reflected the complications in the architecture of small intestine including the loose structure of the mucous membrane (L), shortened and fractured villa (S), and infiltration of the inflammatory cells into the lamina propria (F).

In addition, intestine showed cystic dilatation of some intestinal glands (C) (Figure 3 C).

DNA fragmentation
Figure (4) showed that CCl₄ produced marked intestinal DNA fragmentation below 50bp indicating the high apoptotic damaging effect of CCl₄ compared to control and WGO groups. On the other hand, much less smear of fragmented DNA was detected in CCl₄ + WGO group when compared with intact DNA in the control group.
CCL₄ was used for many years in experimental rodent models to induce hepatotoxicity at different levels including liver injury, fibrosis, cirrhosis, necrosis and hepatocellular carcinoma (HCC). The model of acute CCL₄-induced necrosis in the liver has been investigated after oral administration of CCL₄ (LeSage et al., 1999). CCL₄ is chemically lipophilic and diffuses simply across the cell membranes then gets distributed into the tissues (Ritesh et al., 2015). In CCL₄ intoxicated mice, damage to the endoplasmic reticulum (ER) and accumulation of lipids occur in the hepatic cells (Jeon et al., 2003). Boll et al. (2001) reported that an increase in the esterification of free fatty acids of triglycerides (TG) and phospholipids occurred due to administration of CCL₄ which in turn caused a disturbance in normal lipid homeostasis and consequently, lead to increase the levels of cholesterol synthesis.

In the present study, serum levels of total lipids, triglycerides, total cholesterol, LDL, and VLDL were significantly increased, while HDL level significantly decreased with the CCL₄ administration in comparison to control group. These findings were in agreement with Kumar et al. (2007), they reported a hypercholesterolemia in CCL₄ intoxicated models, that was associated with failure of the liver. This condition was due to damage of the hepatic parenchymal cells that led to disturbance of total lipid metabolism in the liver (Raju et al., 2003). These effects were significantly reversed by oral administration of WGO to nearly the normal level in comparison to CCL₄ treated group. WGO is considered as a valuable source of monounsaturated and polyunsaturated fatty acids, including linoleic acid (omega-3 and omega-6) (Said et al., 2008). The monounsaturated fatty acid can reduce serum TG and phospholipid levels (Jenkins et al., 1999). In addition, WGO has a number of components with nutritional and health benefits, like high content of vitamin E and phytosterol.

Those components might be the reason of decreasing the levels of both triglyceride and phospholipid. Moreover, WGO elevated the level of HDL-cholesterol that picks up and transports cholesterol in the blood back to the liver. That in turn leads to its elimination from the body and consequently reduces the harmful effects of LDL-cholesterol and decreases the incidence of atherosclerosis and cardiovascular diseases (Jonnala et al., 2005).

Multiple organ injuries are frequently assessed by the release of lactate dehydrogenase (LDH) activity in plasma. This study showed that LDH is elevated by 1.6 folds in mice subjected to CCL₄ when compared to the control group. This finding may suggest that CCL₄ not only damages the liver but also affects other organs. Administration of WGO might be responsible for the decrease of LDH level leakage, reduce the cell death and protect against organ damage induced by CCL₄ (Zhu et al., 2013). Previous study revealed that oxidative stress in the intestine of the cirrhotic liver might developed either by the generation of ROS in the mucosa or by possible inducers generated in the liver and transported into the intestinal lumen through the bile (Anup et al., 1999). The present study showed that even acute dose of CCL₄ could generate marked (ROS)–induced intestinal damage through Xanthine Oxidase which is abundantly present in the intestinal mucosa (Anup et al., 1999).

CCL₄ rapidly reaches the liver where it metabolized by cytochrome P₄₅₀. It generates the highly reactive free radical CCL₃ that initiates progression of lipid peroxidation of endoplasmic reticulum cell membrane that consequently, causes the formation of malondialdehyde (MDA) that in turn leads to damage in different organs (Ganie et al., 2011). The increase in MDA level in the small intestine leads to failure of the antioxidant mechanisms to prevent the generation of excessive free radicals through reduction of glutathione and superoxide dismutase in liver injury after administration of CCL₄ (Ramachandran et al., 2002).

GSH is a tripeptide thiol that is abundant in the tissue and plays a vital role in protecting cells against peroxides and free radicals generated from toxic substances including CCL₄ (Endo et al., 2013).

GST plays a significant role in cell defense against ROS-mediated injury by detoxification of lipid hydroperoxides that have been developed due to oxidative damage (Yang et al., 2001). The antioxidant enzymes such as CAT, SOD and non-enzymatic electron receptors such as GSH are affected and used as indexes to evaluate the level of oxidative stress. Ramachandran et al. (2002) mentioned that the reason of intestinal oxidative stress in liver treated with CCL₄ are the increase in mucosal xanthine oxidase activity in conjunction with the decrease in the activity of antioxidant enzymes SOD and CAT. This was found in harmony with (Yang et al., 2001) who informed that injection with CCL₄ induced significant reduction in GST that can initiate lipid peroxidation and DNA damage. The current study showed a significant increase in intestinal NO level after oral administration of CCL₄ that reflects the imbalance between pro-oxidant and antioxidant activity which led to tissue injury (Hamouda et al., 2016).

In this study, acute dose of CCL₄ caused a significant reduction in GSH, GST, SOD and CAT levels in the intestinal mucosa, along with a significant elevation in MDA and NO levels. The administration of WGO suppressed the generation of free radicals and increased the levels of intestinal endogenous antioxidants. The ameliorative effect of WGO is due to its high content of vitamin E that has the potency to inactivate the reactive free radicals, thus avoiding the propagation of the radical chain reaction (Ismael et al., 2014). WGO contains a high percentage of unsaturated (81%) and numerous saturated fatty acids 64% that has anti-inflammatory properties and can decrease oxygen free radicals and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (Abd-El-Hameed et al., 2013). In addition, the phenolic compounds in WGO exhibit an antioxidant influence that could reduce the pro-oxidative state and give powerful antioxidant protection to different organs in the body (Durak et al., 2010).

CCL₄ induced damage by over production of lipid peroxidation and generation of reactive oxygen species that attack and damage DNA leading to DNA hydroxylation. Several studies proved that the administration of CCL₄ caused apoptosis and DNA damage in the liver (Wiseman et al., 1996; Aksit et al., 2014). Our study demonstrated that CCL₄ administration caused marked
oxidative DNA damage in the small intestine through overproduction of ROS. WGO administration caused significant reduction of DNA fragmentation as a result of the ameliorative effect of WGO against ROS induced by CCl₄ treatment.

This study showed morphological alterations in the architecture of intestinal tissue which reflect the pathophysiologic complications of the small intestine in response to acute administration of CCl₄. Our findings showed a change of the intestinal epithelial barrier as a shortening of villi, cystic dilatation of glandular cells, infiltration of immune cell. The administration of WGO restored the integrity of the epithelial barrier and the healthy architecture of the small intestine through the reduction of the ROS and lipid peroxidation.

CONCLUSION

The results of the present study demonstrate that CCl₄ caused liver hypercholesterolemia and hyperlipidemia as well as an increase in the atherosclerosis index. CCl₄ caused damage to other organs such as small intestine through increasing the ROS, NO, and oxidative stress. WGO administration has the potency to protect not only the liver but also the small intestine in acute CCl₄-induced tissue damage. The valuable effect is chiefly attributed to its mechanism of reducing the lipid profile and suppressing the oxidative stress that caused DNA damage.

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