Hepato-protective potential of carotenoid meso-zeaxanthin against paracetamol, CCl₄ and ethanol induced toxicity

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Hepato-protective potential of carotenoid meso-zeaxanthin [(3R, 3’S)-β-carotene-3, 3’-diol] was studied using in vivo rat models. Paracetamol (3 g/kg body wt, orally), 20% ethanol (7.5 g/kg body wt, orally) and CCl₄ (2.5ml /kg, ip) were used as hepato toxins. Levels of marker enzymes of hepatic injury such as serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase and alkaline phosphatase, and serum bilirubin, which were drastically elevated by these hepato toxins were significantly decreased by meso-zeaxanthin pretreatment in a dose-dependent manner. Oxidative stress markers, tissue lipid peroxidation, conjugated dienes and tissue hydroperoxides, were high in the paracetamol treated control group animals, which were lowered by meso-zeaxanthin administration. Level of glutathione and antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase, in liver tissue was increased by meso-zeaxanthin pretreatment compared to control group during alcohol and CCl₄ induced hepatotoxicity. Hydroxyproline, an indicator of fibrosis in liver tissue, decreased remarkably by meso-zeaxanthin administration despite its notable elevation in ethanol treated rats. Histopathological analysis of liver tissue showed the hepatoprotective potential of meso-zeaxanthin.

Keywords: Antioxidant, Carotenoids, CCl₄, Ethanol, Free radicals, Paracetamol

Involvement of free radicals in pathogenesis of several degenerative diseases has been well established. Hepatotoxins like carbon tetrachloride (CCl₄), paracetamol (acetaminophen), ethanol, thioacetamide etc are reported to elicit their deleterious effect by inducing oxidative stress in the body. Since liver is the major organ which metabolizes hepatotoxins through the induction of cytochrome P₄₅₀ enzymes, it is an important target of toxicity of drugs. Some cytochrome P₄₅₀ enzymes generates reactive oxygen species such as O₂⁻ and H₂O₂ during its catalytic cycle. These oxidants produce toxicity by protein oxidation, enzyme inactivation and damage to cell membrane via lipid peroxidation and production of reactive lipid aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4HNE).

Administration of antioxidants, which can scavenge the free radicals, could reduce the hepatic injury. Carotenoids, which are present in fruits and vegetables, have diverse biological actions especially antioxidant activity. meso-Zeaxathin [(3R, 3’S)-β-carotene-3,3’-diol], one of the xanthophyll carotenoids (Fig. 1), which along with lutein and zeaxanthin is concentrated in the macula lutea, an anatomic region of posterior retina, where they constitute macular pigment (MP). meso-Zeaxanthin is the strongest antioxidant of the three and it allows a wide range of blue light filtration. Most of the work on meso-zeaxanthin show that its supplementation reduces age related macular degeneration and cataract. However, there are no reports on the use of this carotenoid in any other degenerative conditions. Present study has been undertaken with the aim to determine the hepato-protective activity of meso-zeaxanthin.

Materials and Methods

Chemicals—Nitroblue tetrazolium (NBT), glutathione (GSH) and 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sisco Research

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Fig. 1—Structure of meso-zeaxanthin(MZ)
Laboratories Pvt. Ltd, (Mumbai, India). Paracetamol was obtained from Variety Pharmaceuticals, Shornur, Kerala. Thiobarbituric acid was purchased from Himedia Laboratories, Mumbai, India. Silymarin bought from Sigma Aldrich (St. Louis, USA). Biochemical kits for determining serum-glutamate pyruvate transaminase (SGPT), serum glutamate-oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), and bilirubin content were supplied by Span Diagnostics Ltd, Surat, India. All other chemicals and reagents used were of analytical grade.

Drug—meso-zeaxanthin (MZ) was supplied by Omni Active Health Technologies Pvt. Ltd, Mumbai. A 5% suspension of MZ was prepared in sunflower oil.

Animals—Male Wistar rats (150-200 g) were purchased from the Small Animal Breeding Station, Kerala Agricultural University, Mannuthy, India and were housed in well ventilated cages under controlled conditions of light and humidity and provided with normal mouse chow (Sai Durga Food and Feeds, Bangalore, India) and water ad libitum. All the animal experiments were done as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, and implemented through the Institutional Animal Ethical Committee of the Research Centre.

Hepatic injury

Paracetamol induced hepatic injury—Male Wistar rats were divided into five groups of six animals each. Group I - Normal without any treatment, group II - paracetamol + sunflower oil, which served as control group, group III - paracetamol + 50 mg/kg body wt MZ, group IV - paracetamol + 100 mg/kg body wt MZ, group V - paracetamol + 250 mg/kg body wt MZ. Animals in the groups II-V received a single dose of CCl4 (2.5 ml/kg body wt, diluted with equal volume of liquid paraffin; ip) to induce acute hepatotoxicity. Oral administration of MZ was started seven days prior to CCl4 injection. After 24h of CCl4 administration, all the animals were sacrificed and collected blood and liver tissues.

Ethanol induced hepatic damage—Male Wistar rats were divided into four groups of eight animals each. Group I - Normal without any treatment, group II - Ethanol + sunflower oil treated control group, group III - Ethanol + 250 mg/kg body wt MZ, and group IV - Ethanol + 250 mg/kg body wt MZ. Animals in the groups II-IV received 20% ethanol (7.5 g/kg body wt, 5 ml in the forenoon and 5 ml in the afternoon; oral) along with different doses of MZ dissolved in sunflower oil (oral, 1.5 ml) for six months. At the end of six months all the animals were sacrificed and collected blood and liver tissues for biochemical analysis.

Analysis of hepatic injury—Serum separated from the blood was used to analyze various marker enzymes like SGPT, SGOT, ALP as well as bilirubin levels using commercially available kits.

Analysis of oxidative stress—A 25% liver homogenate was prepared in 0.1M Tris HCl. Lipid peroxidation (LPO) in the liver homogenate was estimated by thiobarbituric acid method8. Tissue hydroperoxides and conjugated dienes were determined by the modified method of John and Steven7.

Analysis of antioxidant profile of liver tissue—Cytosolic sample of liver homogenate (25%) was prepared by centrifugation at 10,000 rpm for 30 min at 4°C and was used for analysis. Superoxide dismutase (SOD) activity in the homogenate was measured by NBT reduction method6. Catalase (CAT) activity was estimated by measuring the rate of decomposition of hydrogen peroxide at 240 nm11. Assay of glutathione peroxidase (GPx) was done based on oxidation of GSH in the presence of H2O212, and the level of glutathione (GSH) in the tissue homogenate was analysed based on the reaction with DTNB13.

Analysis of hydroxyproline content in liver of fibrotic rats—A portion of liver homogenate (25%) from the ethanol intoxicated rats was hydrolyzed
using 6 N HCl and hydroxyproline content was estimated by the method of Newman and Logan.

Histopathological analysis—A portion of liver was washed in PBS, fixed in 10% formaldehyde solution and then embedded in wax. Sections (4 µm) were taken and stained with hematoxylin-eosin.

Statistical analysis—Statistical evaluation of the data was done by one-way ANOVA followed by Tukey test (post-hoc) using InStat 3 software package. The values have been expressed as mean ± SD.

Results

Effect of MZ on serum marker enzymes and bilirubin levels during paracetamol, CCl₄ and ethanol induced hepatotoxicity—Levels of serum marker enzymes of hepatic injury like SGOT, SGPT and ALP as well as serum bilirubin were elevated significantly in paracetamol, CCl₄ and ethanol alone treated control group animals when compared with normal animals and these elevated levels were decreased to almost normal levels by MZ administration in a dose dependent manner (Table 1).

Effect of MZ administration on antioxidant enzymes and GSH levels in CCl₄ and ethanol induced hepatotoxicity—SOD levels in CCl₄ and ethanol alone treated control animals were low compared to normal groups. There was significant elevation of SOD in MZ pretreated group when compared with control group animals (Table 2). Catalase activity in CCl₄ and ethanol alone treated control animals were decreased compared to normal groups. In animals pretreated with MZ, there was significant increase in catalase activity in a dose dependent manner. GPx levels in CCl₄ and ethanol treated control animals were found to be reduced when compared with normal animals. Decreased GPx levels were significantly increased by MZ pretreatment. Similarly GSH levels in CCl₄ and ethanol alone treated control animals were found to be reduced when compared with normal group. These decreased levels were significantly increased by MZ pretreatment in a dose dependent manner.

Inhibition of paracetamol induced oxidative stress by MZ—Formation of conjugated dienes and tissue hydro peroxides as well as LPO levels in the paracetamol alone treated animals were high compared to normal rats, and these parameters were reduced to normal levels in MZ pretreated groups (Fig. 2).

Effect of MZ administration on hydroxyproline content of the ethanol induced fibrotic rats—Hydroxyproline content in the normal animals was only 2.54±0.16 µg/mg.protein and was elevated to 5.75±2.02 µg/mg.protein by ethanol alone treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SGOT (IU/L)</th>
<th>SGPT (IU/L)</th>
<th>ALP (KA units)</th>
<th>Bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>33.6±3.1</td>
<td>40±11.5</td>
<td>32.4±0.6</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Paracetamol control</td>
<td>385.3±16***</td>
<td>364±32***</td>
<td>120.5±3.8***</td>
<td>2.2±0.1***</td>
</tr>
<tr>
<td>50</td>
<td>206±41.3***</td>
<td>261.7±48.8***</td>
<td>98.2±10.8***</td>
<td>1.3±0.1***</td>
</tr>
<tr>
<td>100</td>
<td>113.3±15.7***</td>
<td>141.3±28***</td>
<td>49.9±0.2***</td>
<td>0.7±0.1***</td>
</tr>
<tr>
<td>250</td>
<td>47.7±9.1***</td>
<td>52.7±2.7***</td>
<td>37.1±1.3***</td>
<td>0.65±0.2***</td>
</tr>
<tr>
<td>Silymarin 100</td>
<td>90±7***</td>
<td>43.8±12.7***</td>
<td>35.8±0.9***</td>
<td>0.61±0.2***</td>
</tr>
<tr>
<td>CCl₄ control Treated (Dose)</td>
<td>620.9±27.4***</td>
<td>517±27.3***</td>
<td>139.5±5***</td>
<td>2.6±0.1***</td>
</tr>
<tr>
<td>50</td>
<td>303.2±61.3***</td>
<td>224.2±27***</td>
<td>81.6±4***</td>
<td>1.5±0.3***</td>
</tr>
<tr>
<td>100</td>
<td>158.9±19.4***</td>
<td>145.6±7.9***</td>
<td>50.5±0.3***</td>
<td>0.8±0.1***</td>
</tr>
<tr>
<td>250</td>
<td>90.7±5.4***</td>
<td>56.9±9.6***</td>
<td>43.1±1.9***</td>
<td>0.6±0.1***</td>
</tr>
<tr>
<td>Ethanol control Treated (Dose)</td>
<td>238.6±21.2***</td>
<td>117±22***</td>
<td>85.9±12.9***</td>
<td>1.3±0.3***</td>
</tr>
<tr>
<td>100</td>
<td>78.6±6.1***</td>
<td>78.2±6***</td>
<td>48.8±3.6***</td>
<td>0.7±0.1***</td>
</tr>
<tr>
<td>250</td>
<td>40.7±4.7***</td>
<td>42.6±3.7***</td>
<td>28.9±0.2***</td>
<td>0.6±0.1***</td>
</tr>
</tbody>
</table>

Values are significant at *** P<0.001 against control groups. Dose indicated as mg/ kg body wt.
in the control animals, indicating a fibrotic response. Elevated levels were decreased to 3.48±0.19 µg/mg.protein and 2.72±0.2 µg/mg.protein by MZ treatment in a dose dependent manner (100 and 250 mg/kg body wt, respectively).

Histopathological analysis of paracetamol alone treated control animals showed portal triads with increased number of lymphocytes, and most of the hepatocytes were vacuolated, central veins and sinusoidal spaces dilated (Fig. 3 b, c). Such damages were less in MZ pretreated animals. The liver section of CCl₄ alone treated control groups showed portal area with haemorrhage and congestion. Hepatocytes showed extensive necrosis, shrunk sinusoidal spaces and haemorrhage in some region. Central venous system also appeared congested and scattered inflammatory cells were seen in the portal area. MZ (250 mg/kg body wt) treated liver section showed normal portal triads. There were increased inflammatory cells in the portal area, many hepatocytes showed vacuolation and the cells appeared larger in size. Sinusoidal spaces appeared compressed and central veins in some places appeared dilated (Fig. 3d, e). Histopathology of ethanol treated control group, showed proliferative fibrosis when compared with MZ (250 mg/kg body wt) treated group indicating the protective role of MZ (Fig. 3 f, g).

**Discussion**

Present study focused on the inhibition of hepatotoxicity by carotenoid (MZ). Paracetamol is a common analgesic/anti-pyretic agent, but an over dose can cause fatal hepatic necrosis. Hepatotoxicity by paracetamol mainly comes from the formation of toxic metabolite (N-acetyl–p-benzo-quinoneimine), by hepatic CYP450. This toxic metabolite covalently interacts with thiol groups in the proteins of liver and consequently stimulates lipid peroxidation.
Fig. 3—Histopathological analysis of rat liver. (a) Normal histology of liver; (b) Paracetamol + sunflower oil treated, indicated dilated central vein; (c) Paracetamol + 250 mg/kg body wt MZ treated; (d) CCl₄ + sunflower oil treated, indicated necrotic changes of hepatocytes; (e) CCl₄ + 250 mg/kg body wt MZ treated; (f) Ethanol + sunflower oil treated, indicated proliferative fibrosis; (g) Ethanol + 250 mg/kg body wt MZ treated (Haemotoxylin – Eosin staining, x 400).
Similarly CCl₄ is the best characterized animal model of xenobiotic-induced free radical-mediated hepatotoxicity. CCl₄ requires bioactivation by CYP450 system (mainly CYP2E1) in liver and yields the reactive metabolic trichloromethyl radical (CCl₃·). CCl₃, in presence of oxygen, is transformed into trichloromethyl peroxyl radical, \( \cdot \text{OOCCl}_3 \). These free radicals can bind with poly unsaturated fatty acids (PUFA), forming alkoxy (R·) and peroxy radicals (ROO·) that generate lipid peroxide which may cause cell membrane damage, alteration in enzyme activity and finally induction of hepatic injury/ necrosis. The largest quantity of evidence of oxidative stress in human liver diseases concerns chronic intoxication with ethanol. The liver injury due to acute or chronic abuse in alcohol intake (steatosis plus necrosis, inflammation and fibrosis in the latter case) has been proved to be dependent on its oxidative metabolism at cytosolic, peroxisomal and or microsomal level.

Hepatic injury leads to disturbances in transport function of hepatocytes resulting in leakage of plasma membrane thereby causing an increased enzyme level in the serum. In the present study, the hepatic damage produced by paracetamol, CCl₄ and ethanol were evident by increased enzyme levels (SGPT, SGOT and ALP) in the serum of control group animals. These elevated levels were decreased to normal levels in the MZ pretreated animals, indicating hepatoprotective potential of MZ.

All the hepatotoxins used in the present study induced ROS production in the body. This led to depletion of antioxidant status of hepatic tissue and induced lipid peroxidative degradation of biomembrane in the control group. MZ pretreatment led to significant increase in antioxidant status of the hepatic tissue and reduced lipid peroxidative damage of liver tissue.

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References