Hepatoprotective Effect of Estradiol and \textit{\alpha}-Lipoic Acid in Rats

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Abstract: Chronic liver diseases are common worldwide and are characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. The aim of the current study was to evaluate the hepatoprotective potential of estradiol and \textit{\alpha}-lipoic acid against carbon tetrachloride (CCl\textsubscript{4})-induced liver damage. Six groups of male Sprague-Dawley rats were treated for 4 weeks as follows: the control group, the group treated orally twice a week with CCl\textsubscript{4} (100 mg/kg b.w), the groups treated intraperitoneally with estradiol (1 mg /kg b.w), the group treated orally with \textit{\alpha}-lipoic acid (50 mg/kg b.w) and the groups treated with estradiol or \textit{\alpha}-lipoic acid plus CCl\textsubscript{4}. The results showed that treatment with CCl\textsubscript{4} induced significant changes in serum biochemical parameters, the histological and histochemical pictures of the liver accompanied by a significant increase in MDA and a significant decrease in SOD and GPx in liver. Whereas, animals treated with estradiol or \textit{\alpha}-lipoic acid were comparable to the control. On the other hand, both of estradiol or \textit{\alpha}-lipoic acid could improve the biochemical parameters, decreased the oxidative stress and improved the histological and histochemical pictures in the liver of rats treated with CCl\textsubscript{4}. It could be concluded that estradiol and \textit{\alpha}-lipoic acid have hepatoprotective effect against CCl\textsubscript{4}-induced hepatotoxicity. This improvement was pronounced in the group treated with \textit{\alpha}-lipoic acid.

Key words: Oxidative Stress • Liver • Estradiol • Alpha Lipoic Acid • Hepatotoxicity

INTRODUCTION

Various enzymatic and non-enzymatic systems have been developed by the liver cell to cope up with the reactive oxygen species (ROS) and other free radicals, when a condition of oxidative stress establishes. However, this defense capacity against ROS may become insufficient [1]. Moreover, most drugs are rendered more hydrophilic by biochemical processes in the hepatocyte, yielding water-soluble products those are excreted in urine or bile [2]. Liver injury is generally indicated by the elevations in serum aminotransferase levels, but increases of far more than three times the upper limit of normal may not lead to clinically significant liver damage. This is because of the great capacity of the liver to heal injury, with the subsequent development of adaptive tolerance, as frequently seen with initial exposure to drugs such as tacrine [3] and isoniazid [4].

There are increasing evidences that free radicals and reactive oxygen species play a crucial role in the various steps that initiate and regulate the progression of liver diseases independently of the agent in its origin [5]. By virtue of its unique vascular and metabolic features, the liver is exposed to absorbed drugs and xenobiotics in concentrated form. Detoxification reactions (phase I and phase II) metabolize xenobiotics resulting in increased
substrate hydrophilicity for excretion. Drug-metabolizing enzymes detoxify many xenobiotics but bioactivate or increase the toxicity of others [6].

Abundant reports in the literature suggested a great role of the female gonad hormone, estrogen in protecting women against Alzheimer’s disease and schizophrenia [7]. It is well established that estrogen replacement therapy has favorable effect on the lipid peroxidation [8]. Furthermore, 17-beta estradiol has a high potential antioxidant effect than other steroid sex hormones [9]. Obviously, raloxifene’s (a selective estrogen receptor modulator) effect suggested primary using for treatment and / or prevention of diseases which can be resulted from oxidative stress in postmenopausal women rather than estradiol itself [10]. According to Liu et al. [11] estradiol metabolites (2-OHE  and 4-OHE) were the best antioxidants since they inhibited the in vitro MDA formation in rat brain homogenates.

Micronutrients supplementation becomes increasingly popular over the past decades. Since the early 1990s, α-lipoic acid (ALA) has been used as a dietary supplement, typically at doses in the range of 100-200 mg/day associated with exhibition free radical scavenging activities in both hydrophilic and lipophilic environments. ALA influences oxidative status by scavenging reactive oxygen species (ROS), regeneration endogenous anti-oxidants, repairing oxidative damage, and chelating metal ions [12]. Recently, antioxidant properties proposed for its important role in ameliorating ischemia [13], cardiovascular disorders [14] and diabetes [15] ALA may act as an anti-obesity agent since ALA suppresses cAMP-activated protein kinase which functions as a fuel sensor in the cell and is activated upon energy depletion. Suppression of cAMP-activated protein kinase in the brain is a signal for filled energy stores and leads to a restriction in food intake [16]. The aim of the current study was to investigate the antioxidative activity and potential protective effect of estradiol and α-lipoic acid against carbon tetrachloride-induced hepatotoxicity in rats.

**MATERIAL AND METHODS**

**Chemicals, Drugs and Kits:** Carbon tetrachloride (CCl₃) was purchased from Morgan Chemical Co, (Cairo, Egypt). Estradiol benzoate (Misr Pharmaceutical Company, Egypt), alpha-lipoic acid (thioctic acid) (Global Napi Pharmaceutical Company, Egypt) were used in the present study. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Lactatedehydrogenase (LDH), Glutathione peroxidase (GPx), and Superoxide dismutase (SOD) was purchased from Randox, (Antrim, UK). Alkaline phosphatase (ALP), was purchased from QCA, (AMPOSTA, Spain). Urea was purchased from Prodia, (Korbach, Germany). Lipid peroxide formation was evaluated as malondialdehyde (MDA) was purchased from Oxis Research™ Co., (USA). Alpha fetoprotein (AFP) was purchased from Monobind Inc, (Lake Forest, USA). All other chemicals were of the highest analytical grade available.

**Experimental Animals:** Three-month old male Sprague Dawley rats (100-150 g) were purchased from Animal House Colony, National Research Centre Dokki, Cairo, Egypt. Animals were maintained on standard lab diet (protein: 160.4; fat: 36.3; fiber: 41g/kg of metabolisable energy =12.08 MJ), and housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12h dark/light cycle) and thermally controlled (25± 1°C) at the Animal House Lab., National Research Centre. After an acclimatization period of 1 week, the animals were divided into six groups (10 rats/group) and housed in filter-top polycarbonate cages. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Center.

**Experimental Design:** Animals within different treatment groups were treated daily for 4 weeks as follow: Group (1), untreated control; Group (2), treated orally twice a week with CCl₃ (100 mg/kg b.w) in corn oil; Group (3), treated intra-peritoneal (i.p) with estradiol benzoate (Est.) (1 mg /Kg b.w) in corn oil; Group (4), treated orally with α-lipoic acid (ALA) (50 mg/Kg b.w); Group (5), treated orally with CCl₃ and i.p injected with estradiol benzoate and Group (6), treated orally with CCl₃ plus alpha lipoic acid.

At the end of the treatment period, all animals were fasted for 12 hr and blood samples were collected from the retro-orbital venous plexus from each animal under ether anesthesia according to the method of Cocchetto and Bjornsson [17]. Blood samples were left to clot and the sera were separated using cooling centrifugation at 3000 rpm for 15 min and stored at -20°C until analysis. The following biochemical parameters were assayed in serum: ALT, AST [18], ALP [19], LDH [20], Urea [21], creatinine [22] and AFP [23].

After the collection of blood samples, all animals were killed by cervical dislocation and sample of each liver was weighed (approximately 0.05-0.1 g) and homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate.

[24]. This homogenate was centrifuged at 1700 rpm at 4°C for 10 min; the supernatant was stored at -70°C until analysis. This supernatant (20%) was used for the assessment of GPx [25], MDA [26] and SOD [27]. Another sample of each liver was removed and placed in 10% of natural formalin for histological and histochemical [28].

**Statistical Analysis:** All data were statistically analyzed by analysis of variance (ANOVA) using the General Linear Model Procedure of the Statistical Analysis System [29]. The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio [30]. All statements of significance were based on probability of \( P < 0.05 \).

**RESULTS**

The effects of different treatment on serum biochemical parameter (Table 1) revealed that ALT, AST, ALP, LDH, urea and creatinine were significantly increased in the group treated with CCl4. Animals treated with estradiol or \( \alpha \)-lipoic acid showed a significant decrease in ALP and a significant increase in LDH compared to the control group whereas; the other biochemical parameters were comparable to the control. The combined treatment of CCl4 and \( \alpha \)-lipoic acid or estradiol showed a significant improvement in all serum biochemical parameters tested although they were still differ significantly than the control.

Animals treated with CCl4 showed a significant decrease in GPx and SOD accompanied with a significant increase in MDA (Table 2) however these parameters were comparable to the control when animals treated with estradiol or \( \alpha \)-lipoic acid. Animals received the combined treatment of CCl4 and estradiol or \( \alpha \)-lipoic acid showed a significant improvement in GPx, SOD and MDA towards the control levels although these treatments did not normalize them. It is of interest to mention that this improvement was pronounced in the group treated with CCl4 plus \( \alpha \)-lipoic acid.

The biochemical results obtained in the current study were confirmed by the histological and histochemical studies. The histological examination of the liver sections for the rats in the control group revealed normal hepatocytes architecture and the central vein (Fig. 1A). The liver of the animals treated with CCl4 showed that periportal necrosis and lymphocytic infiltration diffuse between hepatocytes. The hepatocytes are damaged in the form of fatty degeneration, ballooning and homogenous cytoplasm with some sites of edema (Fig. 1B). The microscopic examination of liver sections of the animals treated with estradiol alone showed that the hepatocytes have eosinophilic cytoplasm, slight interstitial hemorrhage and inflammatory cells (Fig. 1C). Moreover, the liver of the animals treated with \( \alpha \)-lipoic acid showed normal hepatocytes with eosinophilic cytoplasm and normal nuclei (Fig. 1D). The histological examination of the animals in the group treated with estradiol plus CCl4 showed restoration of the liver tissue architecture and the distribution of fatty damage around the blood vessels areas (Fig. 1E). The liver tissues of the animals treated with \( \alpha \)-lipoic acid plus CCl4 showed normal hepatocytes but few fatty droplets and fibrous tissues are still around the blood vessels (Fig. 1F).

The histochemical examination of the control group revealed a minimum amount of connective tissues around the central vein (Fig. 2A). The liver of the animals treated with CCl4 alone showed congestion and bundles of

**Table 1: The effect of different treatments on serum biochemical parameters**

<table>
<thead>
<tr>
<th>Groups Parameter</th>
<th>Control</th>
<th>CCl4</th>
<th>Est</th>
<th>ALA</th>
<th>CCl4 + Est</th>
<th>CCl4 + ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/ml)</td>
<td>35.32 +3.17</td>
<td>146.74 +4.14</td>
<td>32.77 +3.25</td>
<td>31.3 +2.54</td>
<td>66.76 +5</td>
<td>61.17 +6.73</td>
</tr>
<tr>
<td>ALT (U/ml)</td>
<td>4.23 +0.707</td>
<td>28.41 +1.87</td>
<td>4.48 +0.57</td>
<td>4.65 +1.14</td>
<td>14.9 +1.39</td>
<td>16.05 +0.87</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>54.43 +4.64</td>
<td>145.28 +9.72</td>
<td>36.78 +1.74</td>
<td>38.63 +1.58</td>
<td>57.84 +2.99</td>
<td>71.59 +2.91</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>343.75 +25.59</td>
<td>717.49 +23.36</td>
<td>420.27 +50.03</td>
<td>473.48 +19.97</td>
<td>555.63 +15.46</td>
<td>588.09 +19.1</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>63.95 +0.54</td>
<td>144.66 +10.69</td>
<td>59.82 +1.39</td>
<td>65.64 +4.33</td>
<td>61.87 +2.71</td>
<td>100.67 +7.22</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>5.14 +0.32</td>
<td>24.63 +1.41</td>
<td>8.46 +0.51</td>
<td>11.59 +1.11</td>
<td>14.57 +1.28</td>
<td>15.75 +0.83</td>
</tr>
<tr>
<td>AFP (ng/ml)</td>
<td>1.3 +0.23</td>
<td>4.72 +0.84</td>
<td>1.13 +0.1</td>
<td>1.17 +0.08</td>
<td>3.44 +0.35</td>
<td>1.96 +0.15</td>
</tr>
</tbody>
</table>

Within each row, means superscript with different letters are significantly different \( (P<0.05) \).

**Table 2: The effect of different treatments on liver antioxidant enzymes and lipid peroxidation**

<table>
<thead>
<tr>
<th>Groups Parameter</th>
<th>Control</th>
<th>CCl4</th>
<th>Est</th>
<th>ALA</th>
<th>CCl4 + Est</th>
<th>CCl4 + ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/mg) liver protein</td>
<td>276.72 +4.04</td>
<td>152.38 +3.25</td>
<td>273.22 +3.16</td>
<td>278.41 +1.95</td>
<td>204.42 +8.98</td>
<td>240.91 +3.57</td>
</tr>
<tr>
<td>GPx (U/mg) liver protein</td>
<td>1.05 +0.03</td>
<td>0.36 +0.02</td>
<td>1.08 +0.04</td>
<td>0.96 +0.03</td>
<td>0.71 +0.04</td>
<td>0.63 +0.04</td>
</tr>
<tr>
<td>MDA (nmol/mg) liver protein</td>
<td>31.51 +0.99</td>
<td>81.27 +3.47</td>
<td>37.44 +1.71</td>
<td>34.61 +1.5</td>
<td>54.05 +2.52</td>
<td>64.71 +2.29</td>
</tr>
</tbody>
</table>

Within each row, means superscript with different letters are significantly different \( (P<0.05) \).
Fig. 1: Photomicrographs of a liver section of (A) control rat showing the normal hepatocytes architecture and the central vein, (B) rat treated with CCl₄ showing the periportal necrosis and lymphocytic infiltration diffuse between hepatocytes. The hepatocytes are damaged in the form of fatty degeneration; ballooning and homogenous cytoplasm and sites of edema are seen, (C) rat treated with estradiol alone showing hepatocytes have eosinophilic cytoplasm, slight interstitial hemorrhage and inflammatory cells, (D) rat treated with α-lipoic acid showing normal hepatocytes with eosinophilic cytoplasm and normal nuclei, (E) Rat treated with estradiol plus CCl₄ showing restoration of the liver tissue architecture and the distribution of fatty damage around the blood vessels areas and (F) rat treated with α-lipoic acid plus CCl₄ showing normal hepatocytes but few fatty droplets and fibrous tissues are still around the blood vessels.

Fig. 2: Photomicrographs of a liver section of (A) control rat showing a minimum amount of connective tissues around the central vein, (B) rat treated with CCl₄ showing congestion and a bundles of fibrous tissue surrounding the portal tracts and dissecting the parenchyma, (C) rat treated with estradiol showing congestion and minimum collagenous fibers around the central vein, (D) rat treated with α-lipoic acid showing more or less collagenous fibers in the central vein and blood sinusoids as in control, (E) rat treated with estradiol plus CCl₄, showing congestion and collagenous fibers in the central vein and radiating to blood sinusoids and (F) rat treated with α-lipoic acid plus CCl₄, showing congestion and thickening of the central vein. (Masson’s trichrom X 300)
fibrous tissue surrounding the portal tracts and dissecting the parenchyma (Fig. 2B). Liver sections in the group treated with estradiol alone showed moderate congestion and minimum collagenous fibers around of the central vein (Fig. 2C). Liver sections of the animals treated with α-lipoic acid showed more or less collagenous fibers in the central vein and blood sinusoids as in control (Fig. 2D). The histochemical examination of the liver sections of the animals treated with CCl₄ plus estradiol showed congestion and collagenous fibers in the central vein and radiating to blood sinusoids (Fig. 2E). However, the liver sections in the animals treated with CCl₄ plus α-lipoic acid showed congestion and thickening of the central vein and bundles of collagen fibers in blood sinusoids were also seen (Fig. 2F).

**DISCUSSION**

Liver has a unique vascular and metabolic architecture, which is exposed to absorbed drugs and xenobiotics in concentrated form. The current study was conducted to evaluate the protective role of estradiol and α-lipoic acid in rats intoxicated with CCl₄. The selective dose of CCl₄, estradiol and lipoic acid were literature based [31, 32] respectively. The current study revealed that treatment with CCl₄ caused significant increases in ALT, AST, ALP, LDH, urea, and creatinine levels. These results may indicate degenerative changes and hypofunction of liver and kidney [33-35] as well as hepatic cell destruction [36] which increased the release of these enzymes in the blood stream [37]. Moreover, the increased level of LDH in the CCl₄-treated group is an indication of CCl₄-induced myocardial infarction and cardiac injury [34]. These results clearly demonstrated that CCl₄ has a harmful and stressful influence on the hepatic, renal, and cardiac tissue consistent with those reported in the literature [38]. It has also been reported that systemically administered CCl₄ in rats was distributed at higher concentrations in the kidney than in the liver [34]. According to Abraham et al. [39], the kidney has an affinity for CCl₄ and it contains cytochrome P450 predominantly in the cortex [40]. Accordingly, the mechanism of CCl₄ nephrotoxicity is probably the same as that of the liver and also independent from the diminished functionality of the liver.

The current study revealed that CCl₄-induced oxidative stress through significant reduction of GPX and SOD and the elevation of MDA. MDA is an end product of lipid peroxidation and is widely used as a marker of lipid peroxidation. Lipid peroxidation (LP) is one of the main manifestations of oxidative damage and it has been found that it plays an important role in toxicity and carcinogenicity [31, 41-44]. Alteration in the hepatic antioxidant status may therefore be manifestation of oxidative stress caused by CCl₄ and its metabolites. Both GPX and SOD are considered enzymatic free-radical scavengers in cells. It is well known that SOD plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues [43]. Moreover, SOD removes superoxide by converting it to H₂O₂, which can be rapidly converted to water by CAT [44]. Taken together, the increased level of MDA and the decreased activity of antioxidant enzymes GPX and SOD may be attributed to free radical formation which initiated chain reactions of direct and indirect bond formation with cellular molecules (nucleic acids, proteins, lipids and carbohydrates) impairing crucial cellular processes that may ultimately culminate in extensive cell damage and death [45].

AFP is considered specific biomarkers for liver cancer and it is synthesized mainly in the fetal stage; practically no production of this marker occurs in the normal adult. However, when some adult cells are transformed to cancer cells, the synthesis of AFP commences again. The present results revealed that AFP was significantly increased in CCl₄-treated group. The increased level of AFP reported in the current study revealed that CCl₄ is not only hepatotoxic agent but also a cancer promoter. Similar to these observations, Takahashi et al. [46] reported that AFP was significantly increased in CCl₄-treated rats. In the same regards, Frezza et al. [47] reported that intragastric administration of CCl₄ for 30 weeks induced liver cirrhosis and hepatocellular carcinoma.

In the current study, animals treated with estradiol alone showed a significant increase in LDH, creatinine and MDA accompanied with a decrease in ALP activity; whereas, the other biochemical parameters were comparable to the control. Similar to the current results, Nagy et al. [48] reported that estrogen increased LDH activity. Administration of estradiol to CCl₄-treated rats succeeded to restore the changes in all biochemical parameters towards the normal values of the control. According to Shen et al. [49], the mechanism by which estradiol induced its hepatoprotective properties may be due to the fact that estradiol induced the expression of heat-shock protein 70 (Hsp70), decreased NO, enhanced antioxidant enzyme activities, reduced neutrophil infiltration, and reduced lipid peroxidation. It was well established that Hsp70 was up-regulated after ischemic insult, and it could prevent both cardiac and hepatic injuries. De Van et al. [50] reported that NO production in
stressed cells could be modulated by the heat shock response (HSR); through repression of iNOS gene transcription after heat shock, Hsp70 and other heat-shock proteins may play a role in mediating iNOS inhibition.

On the other hand, Hsp70 over expression could protect cells from stress-induced apoptosis and it could affect multiple apoptotic pathways [51]. Hsp70 had been demonstrated to affect processes regulating apoptotic signaling, effectors molecule activation, and downstream of caspase activation [52]. Moreover, Hsp70 could prevent apoptosis by affecting both upstream signaling (SAPK/JNK activation) and downstream effector (caspase-3-mediated) events [53]; and it also could prevent cell death and apoptosis by interfering with the ability of cytochrome c and Apaf-1 to recruit procaspase-9 [54]. Another mechanism by which estradiol may induce its hepatoprotective effect may be due to the ability of estradiol to increase antioxidant enzyme expression in heart, kidney, and liver cells and protecting cell function under different condition [55]. The results of the present study showed that treatment with estradiol could increase total SOD activity and decrease MDA concentration in CCl₄-treated rats. These results suggested that estradiol treatment could also increase antioxidant bioactive molecule expression in liver after CCI₄ intoxication and attenuate neutrophil infiltration and ROS injury in liver or other tissues [56].

In the present study, animals treated with α-lipoic acid were comparable to the controls except a slight decrease in ALP accompanied with a slight increase in LDH and MDA. However, animals received the combined treatment of CCl₄ and α-lipoic acid showed significant improvements in all tested parameters although most of them were still significantly different from normal control values. The protective role of α-lipoic acid against hepatotoxicity and oxidative stress is well documented in a series of scientific reports [57]. α-Lipoic acid is a disulfide compound that functions as a coenzyme in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase mitochondrial reactions, leading to the production of cellular energy (ATP). It is well known that α-lipoic acid and its reduced form, dihydrolipoic acid, reduce oxidative stress by scavenging a number of free radicals in both membrane and aqueous domains by preventing membrane lipid peroxidation and protein damage through the redox regeneration of other antioxidants such as vitamins C and E, and by increasing intracellular glutathione [58]. In addition to its role as an antioxidant, α-lipoic acid can affect the activity of enzymes at various levels of metabolic pathways. Hong et al. [59] showed that α-lipoic acid can affect mammalian pyruvate dehydrogenase complex (PDC) based on its stereoselectivity. It has been shown that short-term administration of α-lipoic acid at high dosage to normal rats caused an inhibition of gluconeogenesis secondary to an interference with hepatic fatty acid oxidation [60], and increased plasma levels of pyruvate and lactate were observed in α-lipoic acid treatments [61]. Since it has been found that hepatocyte NO synthesis is closely associated with the lipid or carbohydrate metabolism, intermediates such as pyruvate and lactate can effectively inhibit LPS/ cytokine-mediated NO synthesis in hepatocytes [62]. Therefore, inhibition of NO synthesis by α-lipoic acid improved carbohydrate metabolism of hepatocytes and it may affect protein (enzyme) functions by modifying the SH-group in protein (enzyme).

Another report showed that α-lipoic acid could inhibit cytochrome P 450 reductase from hepatic microsomes through disulfide-thiol exchange between α-lipoic acid and cytochrome P450 reductase [63]. It well known that structure of nitric oxide synthase (NOS) is homologous to cytochrome P450 [64]. Therefore, it cannot be excluded that inhibition of hepatocyte NO synthesis by α-lipoic acid is directly associated with reduced NOS activity due to the disulfide-thiol exchange between α-lipoic acid and NOS.

The histological and histochemical results confirmed our biochemical finding. The liver of the animals treated with CCl₄ showed periportal necrosis and lymphocytic infiltration, diffuse between hepatocytes. These observations were also reported previously [65]. Treatment with estradiol also induced a protective effect of the liver tissue as demonstrated by the histological and histochemical study. These findings are in agreement with those reported by Kim et al. [65]. On the other hand, the histological and histochemical results reported in the current study in animals treated with CCl₄ plus α-lipoic acid were in agreement with the results reported previously [66-68].

**CONCLUSION**

It could be concluded that estradiol and α-lipoic acid exhibited antioxidant, free radical scavenging activities and hepatoprotection properties against CCl₄-induced liver injury Moreover, this antioxidative effect was pronounced in the group treated with α-lipoic acid than those treated with estradiol.
REFERENCES


