

CYCLOPHOSPHAMIDE HEPATOTOXICITY: THE ROLE OF 4-HYDROXYNONENAL AND CYTOCHROME C OXIDASE AND THE POSSIBLE PROTECTIVE EFFECT OF *Ganoderma lucidum* EXTRACT

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Abstract: Cyclophosphamide (CPA) is a widely used anti-neoplastic drug, but its usage is associated with several side effects including hepatotoxicity. This study was designed to evaluate the ameliorative effect of *Ganoderma lucidum* (GL) extract as a natural antioxidant against CPA hepatotoxicity in male Wistar albino rats. Four groups of rats (n = 7) were used in this study, Group I: served as a control group, group II: was administrated CPA (5 mg/kg), group III: received GL extract (50 mg/kg), and group IV: received CPA and GL extract at the same doses of groups II and III. All the treatments were orally administrated via gastric intubation for 21 consecutive days. Serum activities of the hepatic enzymes (AST, ALT and GGT) in addition to serum proteinogram were evaluated. Moreover, hepatic oxidant-antioxidant markers (4- hydroxynonenal (4-HNE), GSH, CAT and SOD) besides activity of cytochrome C oxidase enzyme were detected. Also, histopathological examination of the hepatic sections (H&E stained) was performed. Serum activity of liver enzymes was elevated, proteinogram was disturbed in CPA-treated animals which accompanied by an increase in hepatic content of 4-HNE, depletion of hepatic antioxidant (GSH, CAT, SOD) and inhibition of hepatic cytochrome C oxidase activity, also hepatic histopathological disruptions were noticed in this group. All these hepatic disturbances were partially ameliorated upon co-administration of GL extract with CPA. In conclusion, production of 4- HNE and inhibition of cytochrome C oxidase enzyme may play a role in occurrence of CPA hepatotoxicity and GL extract may have an ameliorative role against this toxicity.

Key words: cyclophosphamide; hepatotoxicity; *Ganoderma lucidum*; 4-hydroxynonenal; cytochrome C oxidase

Introduction

Cyclophosphamide (CPA) is a drug with a wide spectrum of clinical uses especially in the treatment of cancer (lymphoma, acute and chronic leukemia, multiple myeloma) and autoimmune diseases such as rheumatoid arthritis

and vasculitis (1). As a chemotherapy, CPA is an alkylating agent depending on formation of phosphoramidate mustard (major chemically reactive CPA metabolite) which brings about interstrand cross-links between opposite DNA strands and hampers their replication and tran-

scription process and this characterizes the clinical activity of CPA (2). Bioconversion of CPA into its metabolites (acrolein and phosphoramidate mustard) leads to the formation of high level of reactive oxygen species (ROS), which disturb antioxidant activity inducing CPA-related tissue injuries (3-5).

Oxidative stress (disruption of redox balance) which generates biochemical and physiological disturbances, mediates the injury of normal tissues (as hepatic cells damage and mutagenicity) and this is considered the major limitation of CPA usage as an anti-neoplastic drug (6,7). Several studies suggest that antioxidant supplementation can influence the response to chemotherapeutic drugs and attenuate the development of their adverse side effects (8).

Ganoderma lucidum (GL) or Reishi is an oriental fungus which has been recognized as a medicinal mushroom for over 2000 years due to its multiple powerful effects (9). It presents in many commercial forms including dietary supplement, tea or powder which are produced from different mushroom parts as fruits, mycelia or spores (10). GL polysaccharides (GLPS) is one of the major active bio-components of GL which have multiple pharmacological effects, such as antitumor (11), and antioxidant activity (12) in a dose dependent manner (13) through direct free radicals scavenging and ability to increase antioxidant enzymes activity (14), immunomodulation (15). It also has a well proved hepato-protective effect against chemical or immune-related hepatic damage (16-19).

This study was an attempt to prove our hypothesis about the inhibitory effect of 4-hydroxynonenal and cytochrome C oxidase enzyme in relation to the occurrence of cyclophosphamide hepato-toxicity and possible attenuating role of *Ganoderma lucidum* against this toxicity in Wistar albino rats.

Material and methods

Experimental animal

Twenty eight adult male Wistar albino rats weighting about 180-200 g were obtained from Alexandria University Research Institute, Egypt and kept under 12 hr-12 hr light/dark cy-

cles. They were allowed to standard commercial pelleted food with water *ad libitum*. The animals were left without any treatment for 10 days for acclimatization. All the experimental procedures of this study were performed according to the instructions of "Care and Use of Laboratory Animals" prepared by Faculty of Veterinary Medicine, Alexandria University, Egypt. The protocol of this study was approved by the Research Ethics Committee of the University.

Drugs

Commercially available CPA tablets (Endoxan[®] 50mg, Baxter Oncology GmbH) and GL whole fruiting body extract (Standardized Reishi, 376 mg capsules standardized to contain 10% polysaccharides, Nature's Way[®], USA) were used to accomplish this study. The accurate doses of the drugs were dissolved in saline solution daily and shortly before administration.

Experimental protocol

The acclimated animals were randomly divided into four equal groups (n =7) and treated as follow: group I (control group): rats received 1 ml saline; group II (GL-treated group): rats received GL extract at a dose of 50 mg/kg body weight, bw (20); group III (CPA-treated group): rats received CPA at a dose of 5 mg /kg bw (21); and group IV (CPA+GL-treated group): rats received CPA and GL extract at the same doses of groups II and III. All treatment were orally administrated by gastric intubation for 21 consecutive days.

Twenty four hours after the last administration and under the effect of light ether anesthesia, blood aliquots were collected from retro-orbital venous plexus and then, the animals were dispatched by cervical dislocation to harvest the liver tissue samples. Serum samples were separated by centrifugation of coagulated blood aliquots for 10 min at 1000 ×g and kept frozen at -4 °C for later analysis.

Serum biochemical analysis

Determination of serum activity of hepatic damage biomarkers enzymes including AST, ALT (22) and GGT (23), in addition to serum

level of total protein (TP) (24), albumin (25) and globulins (26) were evaluated using commercially available kits (Biosystems, Spain).

Liver homogenate preparation and biochemical assays

The liver of each animal was rinsed with 0.9% NaCl and deionized water. The tissues were perfused with phosphate buffer saline (PBS), pH 7.4 containing 0.1 mMethylenediaminetetraacetate (EDTA) to discard any red blood cell or clots. Small piece of each liver (about 1 g) was homogenized in 10 ml ice-cold PBS using Glas-Col® tissue homogenizer and centrifuged at $10,000 \times g$ for 30 min. Supernatant was separated, filtrated and stored at -80°C in Eppendorf's tubes for detection of catalase enzyme (CAT) activity depending on rate of hydrolysis of hydrogen peroxide (27), superoxide dismutase enzyme (SOD) activity which determined as the rate of inhibition of reaction between superoxide with a WST-1 dye to form a colored product (28) and reduced glutathione (GSH) content which based on reduction of 2-nitrobenzoic acid (DTNB) with glutathione (29) using locally available kits (Biodiagnostic, Egypt and Abnova, Taiwan). Protein content of tissue samples was detected spectrophotometrically using Bradford reagent (Sigma-Aldrich, USA) (30).

Detection of hepatic level of 4-HNE and activity of cytochrome C oxidase

According to manufacturer instruction, about 100 mg of hepatic tissues was rinsed with PBS, homogenized in 1 ml of ice-cold PBS and stored overnight at -20°C . Two freeze-thaw cycles were done to break the cell membranes; the homogenates were centrifuged for 5 min at $5000 \times g$. The supernatant was removed and kept at 4°C for assessment of 4-hydroxynonenal level using specific ELISA kits (Mybio-source, USA) in addition to activity of cytochrome C oxidase enzyme using specific assay kits (Sigma-Aldrich, USA).

Histopathological examination

Fixed liver tissues (in 10 % formalin) were sliced into 5 μm thick sections after paraffin

embedding and stained with hematoxylin and eosin (H&E) (31) using light microscope.

Semiquantitative grading system for hepatic histopathological alterations

Briefly, five random fields from each animal liver histopathological sections were examined ($\times 100$), the grade of the detected lesion severity was assessed depending on the percentage of affected area/entire section and recorded as follow: (-): absence of lesion, (+): for mild degree of lesions (5–25%), (++): for moderate lesions degree (26–50%) and (+++): for severe degree of lesions ($\geq 50\%$).

Statistical analysis

The statistical analysis was performed using One-way analysis of variance (ANOVA) to study the effect of different treatment on different studied parameters using analysis SAS system software (32).

Results

Serum biochemical results

As shown in Table (1), serum activities of the hepatic enzymes (AST, ALT and GGT) were significantly elevated in CPA-treated animals ($P < 0.05$) when compared to the control group. Concerning serum proteinogram, none of the treated groups showed any significant changes from control group in serum total protein level ($P < 0.05$), but serum albumin level recorded a significant decrease ($P < 0.05$) in CPA and CPA+GL-treated group when compared to the control group. While, serum globulins level showed a significant increase ($P < 0.05$) in CPA-treated group which was accompanied by a significant decrease in A/ G ratio when compared to the control group.

In comparison with CPA-treated group, co-administration of GL with CPA significantly decreased the evoked serum hepatic enzymes activity, decreased serum globulins level and significantly reversed A/G ratio toward normal control level, but, it did not affect the decreased serum albumin level significantly. Administration of GL extract only to the rats significantly decreased serum activity of different hepatic enzymes (AST, ALT and GGT), ($P < 0.05$) but,

it did not have any significant effect on serum proteinogram of these rats as compared to the control group.

Hepatic antioxidant biomarkers

In comparison with the control group, the liver content of GSH along with hepatic activities of CAT and SOD enzymes recorded a significant decrement ($P < 0.05$) in the CPA-treated group. Fortunately, these assessed hepatic antioxidant biomarkers were significantly increased in CPA+GL-treated animals as compared to CPA-treated rats. As compared to the control group, GL extract successfully and significantly increased ($P < 0.05$) these tested antioxidant biomarkers in hepatic tissues of rats when administrated alone (Table 2).

Hepatic level of 4-hydroxynonenal and activity of cytochrome C oxidase enzyme

In CPA-treated group, the hepatic level of 4-hydroxynonenal was increased significantly which was coupled with a significant decrease in hepatic activity of cytochrome C oxidase enzyme ($P < 0.05$) when compared to the control group. These changes were partially and significantly alleviated when GL extract was administrated with CPA. Compared to the control group, administration of GL extract alone to the rats significantly decreased ($P < 0.05$) liver content of 4-hydroxynonenal and enhanced the activity of cytochrome C oxidase enzyme (Table 2).

Histopathological results

Light microscopy examination of representative histological liver sections of various treated animals groups showed normal and well detailed hepatocytes which were arranged in regular cords around portal vein in area in both of control and GL extract administrated groups (Fig. 1). However, CPA-treated group exhibited severe hemorrhage (Fig. 2) with broadening of portal area with mononuclear inflammatory cell and fibroblast besides vacuolar degeneration of the majority of hepatocytes (Fig. 3), but CPA+GL extract-treated group showed small focus of necrotic hepatocytes with inflammatory cells infiltration (Fig. 4) with minimal infiltration of these inflammatory cells in portal area (Fig. 5).

Table (3) illustrated the results of histopathological evaluation of the hepatic lesions of different groups using simple semiquantitative scoring system which affirmed the alleviating role of GL extract on the detected hepatic lesions.

Discussion

Despite of CPA effectiveness in treatment of a wide variety of neoplastic and autoimmune diseases (1), damage or injury of healthy tissues may be the major factor of limited usage of CPA as an anti-neoplastic treatment (33). CPA hepatotoxicity was reported in several studies

Table 1: The effect of different treatments on serum hepatic biomarkers enzymes (AST, ALT and GGT) and serum proteinogram

| | Control | GL | CPA | CPA+GL |
|-----------------|-------------|-------------|-------------|-------------|
| AST (U/L) | 35.20±2.91c | 31.80±3.43d | 79.80±3.85a | 61.40±3.61b |
| ALT(U/L) | 25.80±3.12c | 22.80±2.63d | 69.80±3.81a | 40.60±3.16b |
| GGT(U/L) | 22.00±2.35c | 19.80±1.32d | 51.60±2.66a | 34.20±2.52b |
| TP (g/dl) | 6.52±0.19ab | 6.50±0.19ab | 6.88±0.13a | 6.02±0.13b |
| Albumin(g/dl) | 4.18±0.14a | 4.22±0.09a | 2.98±0.11b | 3.56±0.05b |
| Globulins(g/dl) | 2.34±0.14b | 2.28±0.11b | 3.90±0.14a | 2.37±0.08b |
| A/G ratio | 1.82±0.12a | 1.87±0.06a | 0.76±0.05c | 1.51±0.07b |

All the values are expressed as mean ±SD. Means within the same raw of different litters are significantly different at ($P < 0.05$). **GL:** *Ganoderma lucidum* extract, **CPA:** Cyclophosphamide, **CPA+GL:** Cyclophosphamide+*Ganoderma lucidum* extract.

Table 2: The effect of different treatments on hepatic antioxidants content (GSH, CAT and SOD), 4-HNE level and cytochrome C oxidase activity

| | Control | GL | CPA | CPA+GL |
|-------------------------------------|-------------|--------------|-------------|-------------|
| GSH (mmol/mg protein) | 79.00±3.18b | 110.60±4.45a | 37.20±2.80d | 55.80±2.84c |
| CAT (U/mg protein) | 10.10±0.87b | 17.00±0.79a | 3.58±0.39d | 7.40±0.43c |
| SOD (U/mg protein) | 9.72±0.82b | 16.04±0.62a | 4.02±0.18d | 6.76±0.40c |
| 4-HNE (pg/ mg protein) | 13.90±1.49c | 8.10±0.64d | 48.40±3.08a | 29.00±2.93b |
| Cytochrome C oxidase (U/mg protein) | 51.20±3.34b | 66.40±3.04a | 25.80±2.22d | 35.40±2.80c |

All the values are expressed as mean ±SD. Means within the same raw of different litters are significantly different at (P < 0.05).

Table 3: The score of detected hepatic lesions in male Wistar albino rats of different experimental groups

| Scored liver lesions | Incidence ¹ and Severity ² of histopathological lesions | | | | | | | |
|--|---|----------|---------------|--------------|----------------------|----------|---------------|--------------|
| | CPA-treated rats | | | | CPA+ GL treated rats | | | |
| | Absent (-) | Mild (+) | Moderate (++) | Severe (+++) | Absent (-) | Mild (+) | Moderate (++) | Severe (+++) |
| 1-Congestion of blood vessels | | 2 | 3 | 2 | 0 | 4 | 3 | 0 |
| 2-Hepatocytes vacuolar degeneration | 0 | 0 | 4 | 3 | 2 | 2 | 2 | 1 |
| 3-Portal Inflammatory cells infiltration | 0 | 1 | 4 | 2 | 1 | 3 | 3 | 0 |
| 4-Hepatocellular necrosis | 0 | 2 | 3 | 2 | 2 | 2 | 3 | 0 |
| 5-Hyperplasia of biliary epithelium | 0 | 3 | 2 | 2 | 0 | 5 | 2 | 0 |
| 6-Hemorrhage | 0 | 4 | 2 | 1 | 7 | 0 | 0 | 0 |

¹Number of rats with lesions per total examined (7 rats).

²Severity of lesions was graded by estimating the percentage area affected in the entire section.

(34-37) as the liver is the primary site for microsomal activation of this drug (38). The prime cause may be reactive oxygen species (ROS) generation including hydrogen peroxide and superoxide anion during CPA metabolism which in role exhaust anti-oxidant mechanisms of liver (3, 39). This action may be exaggerated by CPA reactive metabolite (Acrolin) which can react with hepatic GSH causing its depletion (40) which collectively increase susceptibility of lipid peroxidation and hepatic tissues injury (41).

The previous mechanisms of CPA-induced hepatotoxicity may explain the significant increase in serum hepatic enzymes activity (AST, ALT and GGT) of CPA-treated animals which in a concomitant with decreased activity of CAT and SOD enzymes in addition to depletion of GSH in hepatic tissues of the same group may represent a sufficient indicators for the oc-

currence of oxidative injury of hepatocytes consistently with several previously recorded findings (20, 36, 42). Several studies have reported that GL extract is a good free radical scavenger (13, 43, 44), so treatment with GL rendered some protection against CPA-hepatotoxicity which was evidenced by a significant decrease in serum activity of AST, ALT and GGT which accompanied by a significant increase in hepatic tissues content of GSH and activity of antioxidant enzymes (CAT and SOD) in CPA+GL-treated group when compared to the CPA-treated group. Hepatoprotective effect of GL against oxidative damage was previously detected and proved (18). Albumin is one of plasma proteins which is synthesized by liver so, determination of its levels is considered one of best indicators to evaluate hepatic function efficacy (45) and this may demonstrate its decrease in both of CPA and CPA+GL-treated groups which may be attributed to hepatotoxic

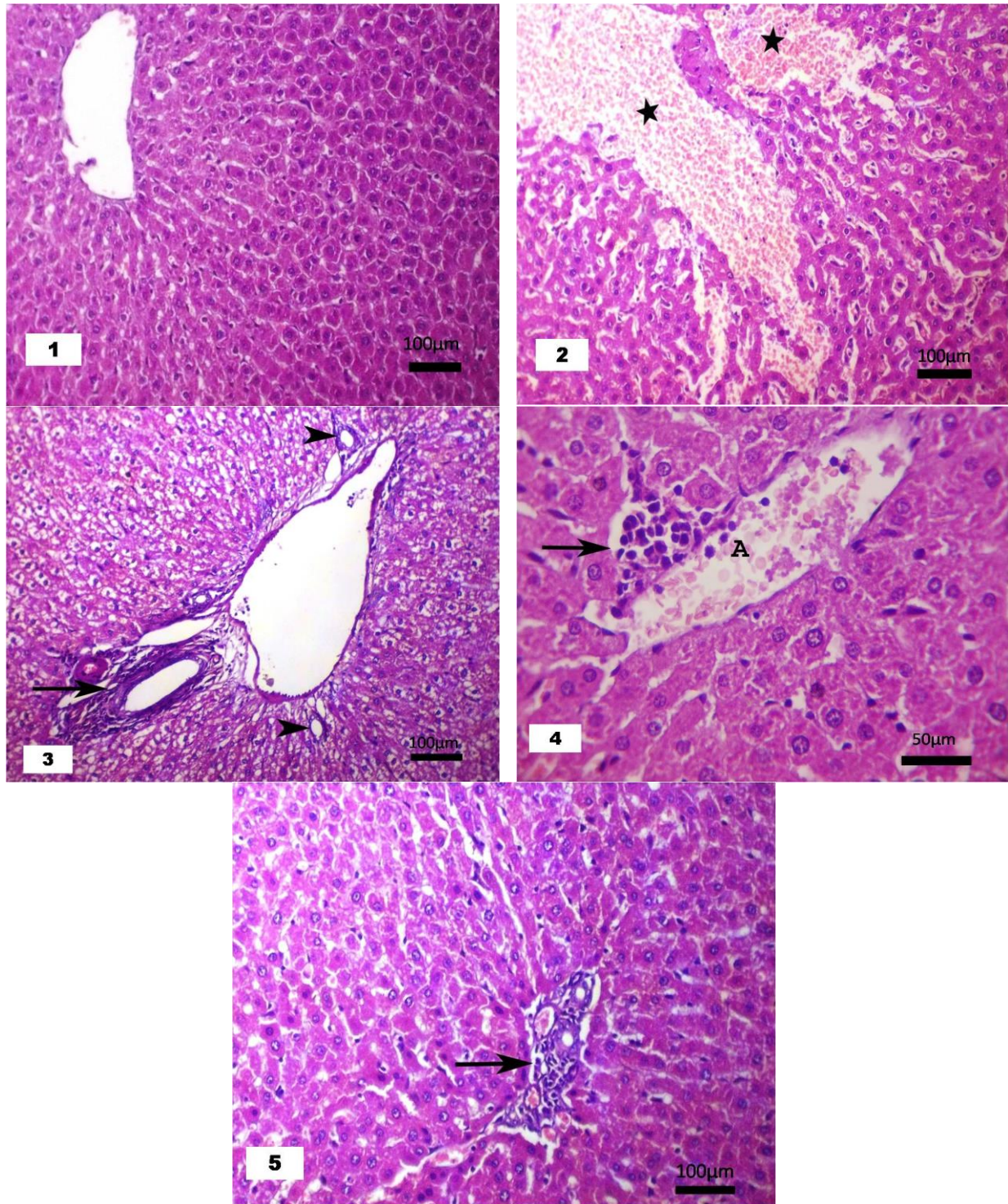


Figure 1: (1) Control group, a section in the liver of a rat showing normal hepatocytes (H&E, X200). (2) CPA-treated group, a section in the liver of a rat showing focal hemorrhage (stars, H&E, X200). (3) CPA-treated group, a section in the liver of a rat showing thickening of portal area with mononuclear cells infiltrate and fibrosis (arrow) and hyperplasia of biliary epithelium (arrow heads) besides hydropic degeneration or vacuolation of most of hepatocytes (H&E, X200). (4) CPA+GL-treated group, a section in the liver of a rat showing congested blood vessel (A) and necrosis hepatocytes with inflammatory cells infiltrate (arrow, H&E, X400), (5) CPA+GL-treated group, a section in the liver of a rat showing mononuclear inflammatory cells in the portal area (arrow, H&E, X200)

impact of CPA, while hyperglobulinemia of hepatocellular disorders, appearing as an inflammatory response to liver injury due to the probable increase in production alpha and beta globulins by liver and/or immune- globulins by plasma cells (45,46). The previous explanation may clarify the reasons for hypoalbuminemia and hyperglobulinemia which significantly decreased A/G ratio of CPA-treated animals which could be attributed to CPA hepatotoxic effects. The treatment with GL significantly ameliorated these changes in proteinogram and clearly lessens the decrease in A/G ratio caused by CPA treatment, thereby GL may exhibit a considerable suppressive effect on hepatic inflammatory reactions caused by CPA, this ameliorative effect of GL on hepatic inflammatory state was concluded formerly (18).

4-hydroxynonenal is the major lipid peroxidation product which is produced in the tissues as a result of cellular exposure to oxidative stress (47). It has a suppressive effect on cytochrome C oxidase enzyme activity (48,49) which is the key enzyme of aerobic cell respiration that contributes to the formation of ATP (essential energy molecules for cell livability and viability), so deficiency of this enzyme represent a risk for the living cells (50). The proved oxidative stress caused by CPA administration may demonstrate the increment in hepatic tissues level of 4-hydroxynonenal which in turn may be the cause for the decrement in these tissues activity of cytochrome C oxidase enzyme. Another explanation for inhibition of hepatic cytochrome C oxidase enzyme in CPA-treated rats may be the great susceptibility of its mRNA as well as protein to oxidative damage (51).

The later changes were alleviated by the dosage of powerful antioxidant GL extract to the rats of the CPA+GL group. Hence, the antioxidant activity of GL was reflected on the effects of its supplementation as a sole treatment to the rats of GL-treated group, as it successfully boosted the hepatic antioxidant biomarkers (GSH, CAT, SOD), which in role, decreased hepatocytes destruction and leakage of hepatic enzymes to the blood and finally decreased hepatic level of 4-HNE and enhanced

the activity of cytochrome C oxidase enzyme as compared to control group.

In this study, histopathological examination of liver, affirmed that CPA can cause damage to the liver which was evidenced by presence of several different hepatic lesions, which might be caused by cellular damaging potential of CPA metabolites in relation to generation of ROS in agreement with the current studies on CPA-induced hepatotoxicity (38,42,52). These histopathological changes correlated strongly with the altered serum enzymes activity and hepatic antioxidants depletion of CPA-treated group. Semiquantitative evaluation of hepatic lesion confirmed the biochemical results which illuminated that the treatment with GL extract effectively ameliorated the abnormal hepatic histopathological lesions caused by CPA, and this may be attributed to its powerful antioxidant ability in prevention of ROS membrane damage (12,16,18).

Conclusion

Our results reported the role of 4-hydroxynonenal production and inhibition of cytochrome C oxidase enzyme in CPA-induced hepatic damage. In addition, it clarified and supported that the treatment with GL extract can reduce oxidative hepato-cyto damage caused by treatment with cyclophosphamide due to its powerful antioxidant effect, so it can be used during treatment with this anti-cancer drug to minimize its deleterious effects on the liver.

Conflict of interest

The authors declare that she has no conflict of interest.

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