Oxidative stress predominates apoptosis during experimental hepatocellular carcinoma

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Introduction

Globally, hepatocellular carcinoma (HCC) ranks the sixth prevalent cancer and the third cause of death related to cancer.1 The well-known risk factors of HCC include untreated viral hepatitis (HBV and HCV), some food flavors, alcohols, aflatoxins, toxic chemicals from industrial processes and environmental pollutants.2 Diethyl nitrosamine (DENA), a strong hepatocarcinogen, was reported induce aberrations in the nuclear enzymes engaged in DNA repair and replication.3 Nitrate and nitrite are used as preservatives in both meat and fish industry, color fixatives and cost effective flavors that can generate endogenous hepatotoxic nitrosodimethylamine oxidative stress markers, as well as, an anti-apoptotic mediator (oncogenic protein Bcl-2), with AFP and histopathology of liver tissue.

Materials and Methods

Chemicals

DENA and the other fine chemicals were obtained from Sigma-Aldrich Chemical Co., USA. Rat AFP and rat Bcl 2 ELISA kits were purchased from TSZ Scientific LLC, USA. Other used chemicals were of analytical grade, purchased from local suppliers.

Animals

We recruited 18 female rats weighing 180 g (±20 g) obtained from the Faculty of Veterinary Medicine, in the University of Cairo, Egypt. They were accommodated for 1 week before treatment success via adjuvant therapeutics with a more selective antioxidant efficacy with the common or new therapeutic protocols of HCC. In addition to liver functions, we will estimate oxidative stress markers, as well as, an anti-apoptotic mediator (oncogenic protein Bcl-2), with AFP and histopathology of liver tissue.
experimental work on standard chow and drinking water in the laboratory of Department of Zoology, Faculty of Science, University of Tanta, Egypt. The temperature was kept at 23 ± 2°C with a approximate humidity of 60% under 12 h/12 h light dark cycle. Animals were classified as two equal groups (9 animals each).

**Experimental Design**

**Group 1 (G1):** Control group, rats were given saline, intraperitoneally (i.p) for 60 days.

**Group 2 (G2):** HCC group, rats were given one dose of diethylNitrosamine dissolved in saline (i.p) in the dose of 200 mg/kg b.w. And after 2 weeks, they were given an activating dose of carbon tetrachloride (CCL4) in olive oil in a dose of 2 ml/kg b.w. (i.p). Euthanization of rats was performed after 60 days from DENA injection.

**Blood and Tissue Collection and Preparation**

By the end of the experiment, animals were left without any chow overnight, weighted and euthanized. Blood was collected retro-orbitally by capillary tubes, left for 10 minutes, centrifuged at 3000 rpm for 10 min and sera were collected and kept in clean stoppered plastic vials at –80°C right analyses of alanine transaminase (ALT), AST, Gamma-glutamyl transferase (GGT), T. bilirubin, AFP and Bcl-2.

Both ALT and AST activities were spectrophotometrically estimated, GGT activity was determined and bilirubin was spectrophotometrically estimated. Quantitative measurement of AFP level in serum was executed by ELISA kit (WKEA Med Supplies Corp, China, code no. WAR-348), following the manufacturers’ instructions. Bcl-2 concentration was computed by commercial kit, following the insert instructions (Bcl-2 kit (biorbyt), Life science (USCNK) Company Inc UK Cat. No Orb52840), by sandwich enzyme immunoassay. The absorbance was measured by ELISA plate reader at 405 nm.

Liver tissue was immediately isolated, cleaned from tissues adhering matters, washed by saline solution, cold by ice, then dried on a filter paper and frozen at −80°C. The liver tissues were homogenized in potassium phosphate buffer (10% W/V, 0.01 M pH 7.4) for estimation of glutathione S-transferase (GST), catalase (CAT) enzyme activities, total thiol (TT), total antioxidant capacity (TAC) and total protein (TP) content. KCl solution (1.15 M) was used for estimation of malondialdehyde (MDA) using homogenizer (Hettich model EBA 12R, Germany).

MDA is an end product, produced by decomposition of unsaturated fatty acids attacked by free radicals. MDA was measured spectrophotometrically. The protein content in the tissues was determined spectrophotometrically. TT was assayed by DTNB. TAC was determined utilizing the ferric reducing antioxidant potential. GST enzyme activity was assayed after formation of adduct, through coupling of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB), as described before. CAT enzyme activity monitored depending on H2O2 decomposition at 240 nm.

**Histological evaluation**

Histological study was applied on serial random liver sections (5-μm thick), using rotary microtome (Litz, Wetzlar, Germany) and were stained with Haematoxylin and Eosin (H&E) staining.

**Statistical analysis**

The results were shown as mean ± SEM. Significance of data variations were assessed by one way analysis of variance (ANOVA), followed by computing t-test, which compare between the two groups, using Graph pad prism software. A value of P < 0.05 was our margin of statistical significance.

**Results**

Table 1 depicted that the group treated with DENA depicted significant up regulation of ALT, AST and GGT activities in plasma (P < 0.001) compared to control. Total bilirubin and AFP levels of this group was significantly elevated (P < 0.001), in relation to control group showing the destructive role of DENA. Conversely, serum Bcl-2 level was significantly depressed (P < 0.001), compared to control. Table 2, which included liver tissue chemistry, showed that DENA injection significantly elevated MDA level (P < 0.001), in relation to control. Both TP (P < 0.01), total thiol (P < 0.001) contents, TAC (P < 0.01), GST activity (P < 0.001) and body weight (P < 0.001) were significantly decreased, while catalase activity was significantly upregulated (P < 0.001), compared to control.

**Table 1. Variations in serum ALT, AST, GGT enzyme activities, total bilirubin, AFP and Bcl-2 levels in female albino rats treated with hepatocarcinogenesis (HCC group), compared to control (Values are expressed as mean ± SEM; number of rats = 9)**

<table>
<thead>
<tr>
<th>Parameters Group</th>
<th>ALT (U/l)</th>
<th>AST (U/l)</th>
<th>GGT (U/l)</th>
<th>T. Bilirubin (mg/dl)</th>
<th>AFP (ng/ml)</th>
<th>BCl2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.5 ± 0.3</td>
<td>93.7 ± 1.1</td>
<td>4.7 ± 0.3</td>
<td>0.172 ± 0.016</td>
<td>178 ± 8.2</td>
<td>458 ± 11.3</td>
</tr>
<tr>
<td>HCC</td>
<td>19.7 ± 0.7***</td>
<td>127 ± 2.9***</td>
<td>13.1 ± 0.4***</td>
<td>0.3 ± 0.02***</td>
<td>277 ± 10.9***</td>
<td>347 ± 9.07***</td>
</tr>
</tbody>
</table>

***, indicates significant, compared to control at P < 0.001; **, significant, compared to control at P < 0.01; *, significant, compared to control at P < 0.05.

**Table 2. Variations in the liver tissue content of MDA, total protein, total thiol, TAC, GST and CAT activities, body weight and relative liver weight in female albino rats treated with hepatocarcinogenesis (HCC group), compared to control (Values are expressed as mean ± SEM; number of rats = 9)**

<table>
<thead>
<tr>
<th>Parameters Group</th>
<th>MDA (nmol/g)</th>
<th>Total protein (mg/g)</th>
<th>Total thiol (mM/g)</th>
<th>TAC (µmol/g)</th>
<th>GST (mol/mg/min)</th>
<th>Catalase (mol/min/g)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>123.1 ± 11.5</td>
<td>91 ± 1.4</td>
<td>41.22 ± 0.80</td>
<td>13.6 ± 0.8</td>
<td>5.41 ± 0.069</td>
<td>2.07 ± 0.06</td>
<td>30.8 ± 1.2</td>
</tr>
<tr>
<td>HCC</td>
<td>230 ± 5.6***</td>
<td>84 ± 0.7***</td>
<td>30.1 ± 0.7***</td>
<td>9.7 ± 0.6**</td>
<td>4.0 ± 0.13***</td>
<td>2.9 ± 0.05***</td>
<td>12 ± 2.4***</td>
</tr>
</tbody>
</table>

***, indicates significant, compared to control at P < 0.001; **, significant, compared to control at P < 0.01; *, significant, compared to control at P < 0.05.
Histological evaluation of liver tissue from experimental groups after H&E staining showed that the control group had normal architecture, large polygonal cells with round nuclei and regular hepatic sinusoids arranged among hepatic cords (Fig. 1A). The surrounding hepatocytes showed pyknotic small peripheral nuclei and acidophilic vacuolated cytoplasm and binuclear cells were observed (Fig. 1B). Stained sections of the DENA-intoxicated group revealed a loss of the normal architecture of the liver, with congestion of the central veins and micronodules of varying sizes containing mononuclear inflammatory infiltration were also observed. Pigmented hyper plastic Kupffer cells were also seen (Fig. 1B*).

**Discussion**

The present observation was carried out to pursue the ability of free radicals to modulate apoptotic behavior in hepatocellular carcinogenesis in an experimental model. AST and alanine transaminase (ALT) are reliable markers for liver damage assessment. Hepatotoxicity disrupts hepatocytes membrane leading to spillage of transaminases into plasma. GGT, shows tissue specific action and is up regulated during many normal and disease conditions, as development and carcinogenesis.

Our study showed that DENA-intoxicated group, had a significant elevation in ALT, AST and GGT activities. This elevation was referred to the potential of DENA to release free radicals which damage cell membranes. Plasma total bilirubin is a sensitive test for the assessment of liver diseases. Plasma total bilirubin was significantly elevated in DENA-injected rats, possibly due to interference with the glucuronidation reaction and liberation of unconjugated bilirubin away from damaged liver tissue. High levels of AFP are suggestive of HCC, and more than 70% of HCC holders show high plasma concentration due to tumor secretion. The up regulation of AFP level observed in DENA-treated animals is suggestive of HCC. In our study, the anti-apoptotic factor, Bcl-2, in the group treated with DENA was significantly depressed. This was reported before in DENA related hepatic cancer, where, the initiation and progression of primary HCC was associated with proliferation and disturbed apoptosis linked to abnormal liberation of Bcl-2 and Bax genes. We noticed that oxidative potential resulted from injection of DENA, manifested as, lipid peroxidation and perturbation of membrane unsaturated fatty acids in the cells might oppose apoptosis.

Oxygen-free radicals hit polysaturated fatty acid terminals in phospholipids. MDA is the famous end product of lipid peroxidation, which affect DNA forming chemical adducts. Lipid peroxidation contributes to endogenous DNA changes in humans leading to cancer and other related diseases. In this study, we found significant elevation in liver content of MDA in group treated with DENA. This was previously observed in liver and lung.

Reactive oxygen species (ROS) generated in cells can damage cell membrane, leading to decrease the cellular protein synthetic function. In our results, TP content in HCC group was significantly decreased compared to control, mostly a result of the cellular damage generated by DENA. This could be confirmed by previous reports.

TT content of the body, including Sulphhydryl (SH) terminals found in protein are sought as major systemic antioxidant in vivo, carried by albumin. They behave as reducing moieties in body compartments. Biosynthesis of both cysteine and glutathione mainly occurs in hepatocytes, however, the rest tissues get thiols through sinusoidal supply into blood, thus any damage in liver tissue will affect its production.

In this study, the significant down regulation of the total non-protein thiols and protein thiols confirmed oxidative stress referred to generated electrophiles by DENA toxicity. This shows an accordance with. TAC possesses superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), macromolecules as albumin, ceruloplasmin and ferritin. TAC constitutes the collective effect of all antioxidants in body compartments. In the current work, TAC was decreased in group treated with DENA.

GST mediates the combination of GSH to many electrophilic compounds, as carcinogens, and native reactive species. These compounds become less harmful than the original phase and are excreted out. In our experiments, we have observed a striking decrease in GST activity in liver after DENA treatment. This was reported earlier, where DENA administration initiated renal carcinogenesis with the same effect on GST activity.

Catalase is a peroxisomal enzyme that mediates breakdown of \( \text{H}_2\text{O}_2 \) into \( \text{O}_2 \) and water. It plays a pivotal role in cellular oxidant protection. Fe-catalase is a tetrameric metalloprotein with protohaem, being the major structural component of the active site and the principal determinant of enzymatic activity. (Print) Our observations depicted that experimental HCC group contained a significant elevation of catalase activity. It seems that CAT activity was increased,
since the level of $H_2O_2$ is elevated in HCC group. Previous report depicted that few human cancer cell lines and tissues produced a high amount of $H_2O_2$ during cancer development.19,34,44

The results of our study indicated significant reduction in body weight gain (BWG) of the group treated with DENA, in respect to control group. This was in accordance with Naura et al.9 who showed that DENA administration, greatly decreased anima total body weight. This was, in part, referred to the fact that, cancerous tissue and severe inflammation of hepatocytes may depress the muscle mass formation.43

Conclusion
We conclude that, disturbed antioxidant status, liberation of harmful-free radicals, concomitant to depressed cellular anti-oxidant potential may depress the apoptotic machinery, leaving a free tendency of hepatocytes to carcinogenesis. The elevated oxidative stress, shown by depressed antioxidant mediators plus elevated stress inducers, parallel to the decreased Bcl-2 level, points for assuming that, the oxidative stress contributes priorly role in hepatocarcinogenesis than the pro oncogenic Bcl-2. This promotes diagnosis and treatment of cancer, relying on the studied dilemma of variables, without ignoring oxidative stress at any stage.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; TP, total protein; ROS, reactive oxygen species; GST, glutathione-s-transferase; CAT, catalase; SH-groups, sulfhydryl groups; DENA, diethyl nitrosamine; TAC, total antioxidant capacity; MDA, malondialdehyde; T.Bil, total bilirubin; GGT, gamma glutamyl transferase; BWG, body weight gain, AFP, Alpha feto protein. Bcl-2, B cell lymphoma 2, TT, total thiol.

Author Contributions
Nabil contributed to conception, design, critical review of the manuscript, Afrah shared analysis and point concepts, mostafa shared drafting and review the manuscript, Naglaa drafted the manuscript and Asmaa executed the experimental work and drafted the article.

Declaration of Conflicting Interests
The authors declare that no conflicts of interest regarding publication of the manuscript.

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Ethical Approval
All implemented steps in this work complied with the ethical standards of the University Research Committee and with the Helsinki declaration; no formal ethical review was required.

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References
1. These, ND, Chen CJ, Kew MC. Liver Cancer; Lyon. 2014:577–593.
24. Graph P. Graph Pad Instate Soft Ware. www.graphpad.com.
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