Possible Therapeutic Effect of Arsenic Trioxide and L-Carnitine on Hepatocellular Carcinoma Induced in Rats

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ABSTRACT

The present study aimed to evaluate the therapeutic effect of arsenic trioxide (AS₂O₃) and L-carnitine on hepatocellular carcinoma (HCC)-induced in rats using diethylnitrosamine (DENA) followed by carbon tetrachloride (CCL₄). Fifty male (10-12 weeks old) Wistar albino rats were randomly assigned to five groups namely normal control, carcinogenic, cisplatin, AS₂O₃, and L-carnitine groups. Assessed biomarkers included serum alkaline phosphatase (ALP), total bilirubin (tBil), alanine transaminase (ALT), aspartate transaminase (AST), albumin and total protein as hepatocyte integrity parameters, serum tumor marker alpha-fetoprotein (AFP), hepatic malondialdehyde (MDA), glutathione reduced (GSH) and superoxide dismutase (SOD) as oxidative stress biomarkers. Histopathology and immunohistochemistry P53 findings strongly supported results of biochemical estimations. AS₂O₃ and L-carnitine have therapeutic effect on HCC-induced in rats; possibly through enhancement of tumor cells apoptosis by AS₂O₃ and protective effect of mitochondria by L-carnitine.

Keywords: Diethylnitrosamine, Carbon tetrachloride, Hepatocellular carcinoma, Cisplatin, Arsenic Trioxide, L-carnitine.

INTRODUCTION

Carcinogenesis of hepatic tissues occurs through two main mechanisms: (a) cirrhosis associated with hepatic regeneration after tissue damage caused by hepatitis infection, toxins or metabolic influences, and (b) mutations occurring in single or multiple oncogenes or tumor suppressor genes. Both mechanisms have been linked with survival, differentiation, inflammation and angiogenesis¹.

Arsenic trioxide (AS₂O₃) is a Food and Drug Administration–approved treatment for refractory acute promyelocytic leukemia (APL) and has shown preliminary activity in patients with relapsed/refractory multiple myeloma². Several mechanisms of action have been proposed for AS₂O₃ activity, including induction of apoptosis mediated by reactive oxygen species, promotion of cellular differentiation, and inhibition of angiogenesis³. AS₂O₃ has also been shown to reduce migration and invasion of cervical and ovarian cancer cells in vitro⁴. Preclinical studies of AS₂O₃ have shown antitumor activity in murine solid tumor models, including breast, brain, liver, gastric, prostate, renal, and bladder cancer⁵,⁶.

L-carnitine (LC) is a naturally occurring compound, it is available from the diet or synthesized endogenously by skeletal muscle, heart, liver, kidney, and brain, or can be given as a nutritional supplement⁷. It is primarily located in mitochondria and possess potential protective effects against many mitochondrial toxic agents⁸.

L-carnitine is required for the transfer of long-chain fatty acids from the cytosol into the mitochondria of skeletal muscle and cardiomyocytes during the beta-oxidation of lipids for the generation of energy⁹. It has the capacity to control carbohydrate metabolism and to maintain cell membrane structure and cell viability, and it is a cofactor in the oxidation of long-chain fatty acids¹⁰. It also affects several key enzymes involved in protein and lipid metabolism¹¹. In addition, LC is a substance that can act as an antioxidant and free radical scavenger¹².

Cisplatin is one of the principal platinum-derived chemotherapeutic agents to treat. In recent years, several studies demonstrated that cisplatin-induced cytotoxicity is closely related to increased ROS generation¹³,¹⁴. Cisplatin rapidly accumulates in mitochondria and deteriorates the mitochondrial structure and metabolic function¹⁵. This leads to significant changes in the metabolites level related to the tricarboxylic acid cycle (TCA cycle) and glycolysis pathway¹⁶,¹⁷. However, the precise mechanism of cisplatin-induced metabolic toxicity remains elusive.

Based on these findings, the present investigation aims to evaluate the possible therapeutic effect of arsenic trioxide and L-carnitine in comparison with cisplatin, on hepatocellular carcinoma induced in rats using DENA followed by CCL₄. To achieve this goal serum ALP, tBil, ALT, AST, albumin, total protein and AFP were estimated. Hepatic oxidative stress biomarkers, MDA, GSH and SOD were also measured. Moreover, Histopathology and...
immunohistochemistry of P53 were also applied to confirm the laboratory findings.

MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing 180–200g of the same age were obtained from animal house, Faculty of Veterinary Medicine, Cairo University (Cairo, Egypt). Rats were housed in well ventilated opaque propylene cages with free access to standard diet pellets and tap water and were maintained at 22±2°C under light and dark cycle. Animals were allowed to acclimatize for one week prior to the study. All animal handling and experimental procedures were conducted according to the guidelines for Laboratory Animal Center of Faculty of Pharmacy, Beni-Suef University. The experiments were performed with the permission of the Animal Ethics Committee of Faculty of Pharmacy, Beni-Suef University.

Drugs, Chemicals and Reagent kits

Carbon tetrachloride, diethylnitrosamine and arsenic trioxide were purchased from Sigma Chemical Company (St Louis, MO, USA). L-carnitine and cisplatin were obtained from El Azaby pharmacy (Giza, Egypt). All other chemicals were obtained from certified local sources and were of analytical grade.

ALP, tBil, ALT, AST, albumin and total proteins kits were obtained from Bio Diagnostic Company (Cairo, Egypt). AFP ELISA kit was purchased from Wkea Med Supplies Company (China).

Experimental Design

Fifty male Wistar albino rats weighing 180–200 g (10–12 weeks old) were used. The animals were divided into five groups with 10 animals in each group as follows:

Group I, this group served as negative control group and received only vehicles.

The other four groups were given a single IP injection of DENA (200 mg/kg b.wt.) then after two weeks received a weekly SC. injection of CCL₄ (3 ml/kg b.wt.) for six consecutive weeks.

Group II, this group received DENA followed by CCL₄ and served as carcinogenic group.

Group III, Rats were treated with cisplatin (5 mg/kg, I.P.) for four weeks started after induction of HCC18.

Groups IV, Rats received L-carnitine (1g/kg day, P.O) for four weeks after induction of HCC19.

Groups V, this group received arsenic trioxide (1mg/kg/day, I.P.). After two weeks, administration was changed to twice a week and the total time of drug administration was four weeks 20.

Blood collection and processing of the samples

At the end of the experimental period (12 weeks), blood samples were collected from the retro-orbital venous plexus of rats under light anesthesia. Blood was collected in clean test tubes, allowed to clot, then centrifuged for 10 minutes at 4000 r.p.m. Serum was separated and stored at -20 °C to be used for determination of liver function parameters including ALP, tBil, ALT, ALP, albumin, total protein, and AFP. After collection of blood samples, rats were immediately sacrificed by cervical dislocation and their livers were immediately removed, and were divided into 2 parts; the first parts were kept at -80 °C for estimation of SOD, MDA and GSH levels.

The second parts were preserved in phosphate buffered formalin 10% for further histopathological investigation and immunohistochemical analysis for P53.

Assessment of Biochemical Parameters

Serum ALP, tBil, ALT and AST activities were measured using diagnostic kits as described by manufacturer’s instructions according to the principles described before 21,23. Serum albumin concentration was estimated using Albumin Assay Kit according to the instructions of the supplier. Total proteins were determined according to the methods described previously by 24. Hepatic MDA and GSH content were measured according to the methods of Albro et al.,(1986)25 and Ellman (1959)26 respectively. Hepatic SOD was assayed according to the method of Marklund and Marklund (1974)27. AFP was measured using ELISA diagnostic kits as described by manufacturer’s instructions according to the principle of Gibbs et al.,(1987)28.

Histopathological Examination

Liver tissue specimens were fixed in 10% neutral buffered formalin. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin, sectioned at 4-6U thickness and stained by hematoxylin and eosin (H&E).

Immunohistochemical Staining for p53

The selected paraffin blocks for immunohistochemical staining were sectioned at 5 μm and stained with monoclonal antibodies P53 according to the method described previously29. Each section was counted manually at high power (X400) after identifying at low power (X100) their presentative areas with the highest concentration of stained cells according to the recommendation of Cohen et al30. About 1000 cells/slide were counted in each of five microscopic fields from well-labeled areas to determine the average of P53 labeling index. P53 was expressed as the number of labeled cells (positive staining) as a percentage of the total number of cells counted in each specimen. All identifiable staining was regarded as positive.

Statistical Analysis

Data were presented as mean ± SEM. Statistical analysis of the data was carried out using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test for post hoc analysis. Statistical
significance was acceptable to a level of $p < 0.05$. Data analysis was accomplished using the Statistical Package for Social Sciences (SPSS) software program (version 20).

RESULTS

Effect of Treatments on Hepatocytes Injury Biomarkers

As shown in table 1, carcinogenic group showed a significant increase in serum ALP, tBil, AST and ALT levels. On the other hand there was no significant change in serum albumin and total protein levels in comparison with negative control group. Rats treated with CP, $\text{AS}_2\text{O}_3$, or L-carnitine showed significant reduction in serum levels of ALP, tBil, AST and ALT when compared to carcinogenic group.

Effect of treatments on oxidative stress biomarkers

Carcinogenic group displayed significant elevation of hepatic MDA level coupled with significant reductions of hepatic GSH and SOD levels. Rats treated with L-carnitine revealed significant correction of all oxidative stress biomarkers in comparison with non-treated group. CP and $\text{AS}_2\text{O}_3$ treated groups didn’t show any significant change in hepatic MDA, GSH and SOD levels (Table 2).

Effect of Treatments on Tumor Marker (Alpha-fetoprotein)

As shown in Table 3, HCC was associated with significant increase in AFP level as compared to normal control group. CP, $\text{AS}_2\text{O}_3$, and L-carnitine significantly reduced alpha-fetoprotein level as compared to the carcinogenic group.

Histopathological Findings

Normal control group

Liver showed normal hepatic lobules which are made up of radiating strands of polyhedral hepatocytes vertical to central vein with bile canaliculi between adjacent hepatocytes in the plate. Sinusoids lined by a discontinuous layer of fenestrated endothelial cells Fig. (1A).

Carcinogenic group

Morphologically hepatocellular carcinoma appeared as multifocal, widely distributed nodules of variable size and shapes which embedded in hepatic parenchyma. Histopathological alterations of liver sections were shown obvious fatty degeneration with eccentric nuclei. Some nuclei of hepatic cells were apparently hyperchromatic and displayed some features of pyknosis. Leukocytic infiltration was clearly visible in these group Fig. (1B). Focal neoplastic cells were polyhedral to round with dense, centrally located vesicular nuclei. Hepatocellular carcinomas range from well-differentiated to highly anaplastic undifferentiated lesions. In well and moderately differentiated carcinoma, cells that are recognizable as tissue of origin are disposed either in a trabecular pattern or in pseudoglandular pattern Fig. (1C). poorly differentiated tumor cells showed pleomorphic and numerous anaplastic giant cells were also observed. Fig. (1D).

Cisplatin group

Histopathological findings of liver revealed swelling and vacuolization of hepatocytes. The nuclei appeared vesiculated with prominent nucleoli. Few mitotic figures and hyperplasia of kupffer cells were noticed. Disorganization of hepatic plates that invaded by delicate fibrous connective tissue trabeculae infiltrated with lymphocytes and macrophages were seen. Fig. (1E).

Arsenic trioxide group

Histopathological findings of this group revealed degeneration changes appeared in form of swelling of hepatocytes. Apoptosis of tumor cells displayed as eosinophilic bodies scattered between malignant hepatocytes which showed few mitotic figures with clear pleomorphism. The connective tissue stroma infiltrated with mononuclear cells mainly lymphocytes and macrophages. Fig. (1F).

L-carnitine group

Histopathological findings of liver revealed degenerative changes including swelling and fatty cysts. Focal necrotic areas scatted in the hepatic lobules which infiltrated with mononuclear cells mainly lymphocytes and macrophages. Giant multinucleated cells were seen in little number. Mitotic figures were more prominent than that treated with cisplatin. Pleomorphism of nuclei with clearly numerous karyomegal was observed. Fig. (1G).

Immunohistochemistry study

PS3 immunohistochemical expression of normal control group showed very weak immunostained (less than 5% stained nuclei) Fig. (2A). On the otherside carcinogenic group showed very strong immunostained (more than 60% stained nuclei) Fig. (2B). Group treated with cisplatin showed positive stain in few numbers of hepatocytes nuclei (less than 25% stained nuclei) Fig. (2C). Group treated by arsenic trioxide showed positive stain of little number of hepatic nuclei (less than 30% stained nuclei) Fig. (2D). Liver of group treated with L-carnitine showed positive stain in numerous numbers of hepatocytes nuclei (more than 40% stained nuclei) Fig. (2E).

DISCUSSION

The present study aimed to investigate the protective effects of arsenic trioxide and L carnitine against DENA and CCL₄ induced HCC in rats.

In the present investigations, rats treated with DENA and CCL₄ showed significant histological and biochemical variations, reflecting the instability of liver cell metabolism in addition to distinctive changes in serum enzyme activities and AFP (the relevant tumor marker). DENA is known to cause perturbations in the nuclear metabolism i
enzymes involved in DNA repair/replication and is normally used to induce liver cancer in animal models\(^1\). It is also known that the pathological effects of DENA in the liver is mainly due to its degradation products, either the carbonium ion or the diazoalkane. These two reactive metabolites may react with some vital compounds of the liver, such as DNA or proteins, by alkylation\(^2\).

Induction of hepatocellular carcinoma by DENA and CCL\(_4\) significantly increased serum ALP, tBil, and AST and ALT levels. It is well known that serum levels of AST, ALT, ALP, tBil are indicative for hepatic function and their increase is correlated with the hepatic injury and the disturbance in hepatocytes membrane instability and metabolism\(^3\). The increase of ALT and AST serum levels are specific to hepatocellular disturbance\(^4\). It is well established that ALT level signifies the presence of active disease\(^5\) and is credited to hepatocellular damage and reflects the pathological alteration in biliary flow\(^6\).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal Control</th>
<th>Carcinogenic Control</th>
<th>Cisplatin</th>
<th>AS(_2)O(_3)</th>
<th>L-Carnitine</th>
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<tbody>
<tr>
<td>ALP (U/L)</td>
<td>218.0 ± 20.86</td>
<td>432.2 ± 16.48*</td>
<td>224.3 ± 21.88*</td>
<td>237.2 ± 22.38*</td>
<td>332.2 ± 33.16abc</td>
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<td>tBil (mg %)</td>
<td>0.07 ± 0.01</td>
<td>0.14 ± 0.01*</td>
<td>0.10 ± 0.01*</td>
<td>0.11 ± 0.01b</td>
<td>0.11 ± 0.01b</td>
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<tr>
<td>ALT (U/ml)</td>
<td>47.40 ± 4.61</td>
<td>128.67 ± 2.98*</td>
<td>52.00 ± 3.94b</td>
<td>48.00 ± 4.68b</td>
<td>45.00 ± 2.89b</td>
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<tr>
<td>AST (U/ml)</td>
<td>126.60 ± 10.38</td>
<td>263.00 ± 6.27*</td>
<td>173.60 ± 13.72b</td>
<td>166.70 ± 15.30b</td>
<td>164.50 ± 12.24</td>
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<tr>
<td>Albumin (g/dl)</td>
<td>3.56 ± 0.09</td>
<td>3.33 ± 0.13</td>
<td>3.37 ± 0.17</td>
<td>3.23 ± 0.23</td>
<td>3.33 ± 0.15</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>7.14 ± 0.27</td>
<td>6.90 ± 0.13</td>
<td>6.87 ± 0.37</td>
<td>6.72 ± 0.37</td>
<td>6.45 ± 0.33</td>
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Data were expressed as mean ± SEM (n = 6); Multiple comparisons were done using one-way ANOVA followed by Tukey-Kramer as post ANOVA test; *Significantly different from normal control group at p < 0.05; †Significantly different from carcinogenic control group at p < 0.05; ‡Significantly different from cisplatin treated group at p < 0.05.

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<tbody>
<tr>
<td>MDA (nmol/g)</td>
<td>7.86 ± 0.20</td>
<td>14.04 ± 0.82*</td>
<td>11.83 ± 0.63*</td>
<td>13.92 ± 0.63*</td>
<td>8.26 ± 0.28b</td>
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<tr>
<td>GSH (μmol/g)</td>
<td>7.98 ± 0.57</td>
<td>3.33 ± 0.16*</td>
<td>4.65 ± 0.15*</td>
<td>4.22 ± 0.29*</td>
<td>7.02 ± 0.37b</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>142.4 ± 7.24</td>
<td>106.9 ± 1.36*</td>
<td>112.3 ± 1.93*</td>
<td>109.4 ± 4.74*</td>
<td>135.4 ± 5.27b</td>
</tr>
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<tr>
<td>AFP(μg/L)</td>
<td>1.25 ± 0.03</td>
<td>4.67 ± 0.17*</td>
<td>2.30 ± 0.06b</td>
<td>2.50 ± 0.289b</td>
<td>2.37 ± 0.260b</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SEM (n = 6); Multiple comparisons were done using one-way ANOVA followed by Tukey-Kramer as post ANOVA test; *Significantly different from normal control group at p < 0.05; †Significantly different from carcinogenic control group at p < 0.05.
ALP is a liver function enzyme that relates to the membrane lipid in canalicular ducts. Its increase in serum reflects the biliary flow disturbance and thus the extra or intra-hepatic interference with the bile flow leads to elevation of ALP serum levels. Moreover, Pancoska et al. (2011) showed that ALP levels are elevated in association with small tumors and further increases with increasing tumor mass and thus ALP can be used as a specific tumor marker during diagnosis in the early detection of cancer.

In the current study, the observed increase in serum indices of liver function due to DENA and CCL₄ administration could be a secondary event following lipid peroxidation of hepatocyte membranes, with a consequent increase in the leakage of enzymes from liver tissues. An elevated level of serum indices of hepatocellular damage has been previously reported in many models of DENA-induced hepatocellular degeneration.
The decrease of ALT, AST, ALP and tBil serum levels in AS2O3 rats may be attributed to the decrease of cellular damage. The effect of L-carnitine could be due to stabilization of hepatocyte membranes with the consequent decrease in the leakage of liver enzymes. The interaction of L-carnitine with sarcolemmal phospholipids and mitochondrial membranes has been previously reported.

Oxidative stress, an imbalance between generation of reactive oxygen species and antioxidant defense mechanisms, predisposes to hepatocarcinogenesis and drives HCC in chronic liver ailments. Increased oxidative stress biomarkers and depletion of enzymatic and non-enzymatic antioxidants have been reported in cancer patients and other human diseases.

Data from this study revealed that administration of DENA followed by CCL4 significantly increased hepatic MDA and SOD levels and significantly decreased hepatic GSH level. The contribution of oxidative stress during development of hepatocarcinogenesis and promotion of liver cancer has been recently confirmed. It is well documented that, DENA induces hepatic dysfunction through the induction of disturbances in antioxidant defense systems, increases the reactive oxygen species (ROS) and membrane lipid peroxidation and consequently vital bio-membranes damage. It has been reported that ROS play a major role in tumor promotion through interaction with critical macromolecules including lipids, DNA repair systems and other enzymes. Furthermore, CCL4 is well known to generate peroxy and superoxide radicals which are associated with the inactivation and loss of antioxidant mechanism in animals against oxidative stress.

Our data showed that AS2O3 didn’t significantly alter hepatic MDA, SOD and GSH activities when compared to HCC group. This comes in consistent with previous studies which showed that Several mechanisms of action have been proposed for AS2O3 activity, including induction of apoptosis mediated by reactive oxygen species. Furthermore, arsenic trioxide produces this action by binding protein thiols groups and non-protein thiols such as glutathione (GSH). It also regulates the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and catalase.

On the other hand, the present study demonstrated that administration of L-carnitine completely reversed the increase in MDA and SOD and the decrease in GSH induced by DENA in liver tissues. Our results are consistent with previous studies that have reported that L-carnitine has non-enzymatic free-radical scavenging activity. L-carnitine might also have the activity to improve energy metabolism and repair oxidized membrane/lipid bilayers. Thereby suppressing the release of free electrons from mitochondrial electron transport systems, a prerequisite reaction to generate free radicals. The molecular mechanism by which L-carnitine suppressed hepatic injury and eventually hepatocarcinogenesis should be studied further.

Alpha fetoprotein is a fetal specific glycoprotein secreted from fetal liver and yolk sac, rapidly falls few weeks after birth. AFP is the most important serum marker for diagnosis of HCC. The present study demonstrated that AFP level was elevated in HCC proving the occurrence of premalignant liver changes in DENA treated rats. In the same line of our results Borges et al. and Yeo et al. who reported that, AFP serum level had been elevated in DENA treated rats. Treatment with AS2O3 or L-carnitine showed an ameliorative effect on AFP suggesting an antitumor activity against HCC.

The present work was further supported by histopathological study. Rats injected with DENA followed by CCL4 showed many lesions which manifested the characteristic of malignancy, severe damaged hepatocytes with extensive cytoplasmic vacuolization and hydropic degeneration. Hepatocellular carcinomas range from well-differentiated to highly anaplastic undifferentiated lesions. In well and moderately differentiated tumors, cells that are recognizable as hepatocytic in origin are disposed either in a trabecular pattern or in pseudoglandular pattern. In poorly differentiated forms, tumor cells showed a pleomorphic appearance with numerous anaplastic giant cells. Similar findings have been reported previously. Treatment of rats with arsenic trioxide or L-carnitine showed signs of protection against HCC to a considerable extent.

P53 is an important anticancer gene that is frequently mutated in cancers. Immunohistochemical demonstration of the p53 tumor protein may be useful in predicting prognosis of several types of cancer. The human p53 tumor-suppressor gene, as a transcription factor, plays an important role in the regulation of the cell cycle, maintenance of genomic stability, cell differentiation, and apoptosis. The p53 gene is mutated in about 18–67% of hepatocellular HCC worldwide and plays an important role in the genesis or progression of HCC.

In the present study, P53 immunohistochemical expression of normal control group showed very weak immunostained nuclei. On the other side carcinogenic group showed very strong immunostained nuclei. Arsenic trioxide and L-carnitine groups showed positive stain in few numbers of hepatocytes nuclei. The p53 tumor-suppressor gene has been shown to play a key role in the control of the cell cycle, the maintenance of genomic stability, cell differentiation and apoptosis. In certain conditions resulting in DNA damage, wild-type p53 is activated and results in either G1 arrest or apoptosis of cells. P53 exerts at least part of its function at the transcriptional level through sequence-specific DNA binding and/or interaction with other transcription factors.
CONCLUSION

AS₂O₃ and L-carnitine have therapeutic effect on HCC in experimental rats, mostly due to growth inhibition and apoptosis of malignant cells and protection of mitochondrial and cell membranes. AS₂O₃ is a more powerful than L-carnitine in comparison with cisplatin. These results are promising for further clinical trials on AS₂O₃ and L-carnitine on clinical cases of HCC in humans.

REFERENCES


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