Doxorubicin toxicity can be ameliorated during antioxidant L-carnitine supplementation

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Key words: doxorubicin, L-carnitine, hepatotoxicity, gene expression, antioxidant agent

Introduction

Doxorubicin (Dox) is among the first anthracycline antibiotics clinically used in cancer chemotherapy.1 Its clinical use in long-term treatments is limited by the development of free radicals induced toxicities,2 which are frequently lethal.1,3,4 Its metabolic activity causes generation of free radicals and induction of oxidative stress that correlated with tissue injury.5 Dox causes an imbalance between free oxygen radicals (ROS) and antioxidants enzymes resulting in tissue injury,6,7 that is confirmed by lipid peroxidation and protein oxidation in tissue.8 Dox had toxic effects on liver through increasing levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) enzymes in liver tissue which are found in antioxidant enzyme system.9,10 Regulation of these mediators has been considered for therapeutic necessity to prevent doxorubicin-induced toxicities in various organs.11 L-carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) is a naturally occurring compound that is widely distributed in nature, especially in red meats and dairy products. L-carnitine, an endogenous mitochondrial membrane compound, is a small water soluble molecule. It derived from two sources: endogenous synthesis, low levels of carnitine can be synthesized, primarily in liver and, to a lesser extent, in kidneys and brain, and from exogenous dietary sources.12,13 It is absorbed in the intestine and distributed to various tissues, with skeletal and cardiac muscle stores accounting for more than 98% of the total carnitine pool. L-carnitine presents in both plasma and tissue as free carnitine or bound to fatty acids as acylcarnitine derivatives.14 Its main physical function is facilitating the transport of long chain fatty acids into mitochondria in order to enter the β-oxidation cycle.1,15 By combination with carnitine to form acylcarnitine, acyl groups could be transferred from cytosolic coenzyme A on the outer surface of the mitochondrion membrane, then to the inner surface by exchange with free carnitine using an antiport mechanism. The acyl groups are then transferred from carnitine to coenzyme A within the mitochondrion.15 Carnitine has a protective effect on lipid peroxidation by reducing the formation of hydrogen peroxide16,17 and is associated with buffering of excess acyl-CoA, which is potentially toxic to the cells. It is also improve antioxidant status in rats and showed free radical scavenging activity as well.18 In addition, L-carnitine prevents the accumulation of free fatty acids and their toxic intermediates, thus
The protective effect of L-carnitine on chronic haemodialysis patients and parenteral nutrition.

Carnitine deficiency results in severe cardiomyopathy, congestive heart failure, encephalopathy, hepatic insufficiency, impaired growth and development in children and neuromuscular disorders. 

Exogenous L-carnitine used in myopathies and cardiovascular disease, ischaemic heart disease and chronic haemodialysis patients and parenteral nutrition. 

L-carnitine protected cardiac cells against ischaemia, hypoxia and oxidative stress, by decreasing the levels of toxic acyl-CoA derivatives and regulating carbohydrate metabolism. 

The haemodilution substantially reduces plasma and tissue carnitine levels. L-carnitine shows the most promise among supplement therapies for the management of fatigue in cancer patients. 

In addition, lymphocytes are enriched in carnitine suggesting that the carnitine system might have a considerable role in counteracting the impaired immune responses associated with ageing. 

Carnitine might improve immune system function by its antioxidant action. 

Also, L-carnitine has a protective effect against the toxic actions of different drugs, which induce oxidative stress and/or carnitine deficiency. 

It is used to treat adriamycin-induced cardiotoxicity and valproic acid-induced hepatotoxicity, neurodegenerative disorders, male infertility and AIDS patients and as an additive in extended platelet storages. Therefore, this study has been initiated to study the protective potential of L-carnitine against Dox-induced oxidative hepatocyte injury.

### Results

The cumulative hepatotoxicity of Dox was clearly featured by dose-dependent increase in serum biochemical markers, ALT, ALP and bilirubin (Table 2). The serum biochemical markers were significant increased with doxorubicin dose dependent manner. At the highest cumulative dose, 18 mg/kg, Dox resulted in significant increase by 975%, 114% and 349% in ALT, ALP and Bilirubin respectively, compared to control group. L-carnitine supplementation alone showed no significant changes in biochemical markers. On the other hand administration of L-carnitine in combination with high dose Dox (18 mg/kg) resulted in a complete reversal of Dox-induced increase in serum ALT, ALP and bilirubin to the control values.

Accumulative dose of Dox was resulted in a significant 41% decrease in total carnitine level in liver tissues compared to the control group. Administration of L-carnitine alone resulted in a significant 45% increase in total carnitine level in liver tissues compared to the control group. L-carnitine supplementation alone showed no significant changes in biochemical markers. On the other hand administration of L-carnitine in combination with high dose Dox (18 mg/kg) resulted in a complete reversal of Dox-induced increase in serum ALT, ALP and bilirubin to the control values.

The effect of Dox, L-carnitine and their combination on the oxidative, nitrosative stress biomarkers and indices of lipid peroxidation, TBARS, NOx and GSH in liver tissues are shown in Table 3. The highest cumulative dose of Dox resulted in significant 72% and 84% increase in TBARS and NOx respectively, and a significant 54% and 48% decrease in GSH and SOD respectively compared to the control group. There is no significant change in oxidative, nitrosative stress biomarkers and index of lipid peroxidation observed with L-carnitine supplementation alone. Treatment with L-carnitine resulted in a complete reversal of Dox-induced increase in TBARS and NOx and decrease in GSH in liver tissues to the control values. There is a significant increase of these biomarkers in the started dose of Dox (6 mg/kg) compared to the last cumulative dose.

Table 4 showed that the high dose of Dox resulted in significant decrease of antioxidant enzymes activity of GSHPx, CAT, GR and GST, compared to normal and L-carnitine-supplemented
Doxorubicin is potent anticancer drug, whose clinical use is limited on account of its toxicity. It causes disruption in basal metabolism by showing toxic effect especially in liver and heart tissues in animals. It is known that doxorubicin is considered the most toxic anthracyclines and causes weight loss. It is known that doxorubicin increased serum indices of liver function including ALT, ALP and total bilirubin. These elevations of ALT and ALP are attributed to hepatocellular damage and decreased liver functions. These elevated levels of serum indices for hepatocellular damage has been previously reported in Dox-induced hepatotoxicity model. Interestingly, L-carnitine prevents the increase in hepatic enzymes during its administration in combination with a cumulative dose of doxorubicin. These results suggested that L-carnitine may have protective effect against Dox-induced liver damage. This protective effect might be due to stabilization of hepatocyte membranes by L-carnitine with the consequent decrease in the leakage of liver enzymes.

Our study established that doxorubicin significantly decrease the total carnitine in liver tissues. This could be a secondary effect following inhibition of endogenous carnitine biosynthesis and/or decreased carnitine transport in Dox-induced hepatocytes damage. This hypothesis is consistent with data presented by Laub.

Table 3. The effect of doxorubicin on oxidative and nitrosative stress biomarkers in rats

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>TBARS</th>
<th>NO (x)</th>
<th>GSH</th>
<th>SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>294.5±25.02</td>
<td>39.7±2.49</td>
<td>4.16±0.22</td>
<td>2.4±0.238</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>208.6 ±8.96*</td>
<td>39.1±2.47</td>
<td>4.0±0.43</td>
<td>2.5±0.121</td>
</tr>
<tr>
<td>Doxorubicin 6mg/kg</td>
<td>318.4±5.12*</td>
<td>52.4±1.77*</td>
<td>2.47±0.165*</td>
<td>1.9±0.03*</td>
</tr>
<tr>
<td>Doxorubicin 12 mg/kg</td>
<td>332±18.59*</td>
<td>56.5±3.2*#</td>
<td>2.30±0.147*</td>
<td>1.18±0.09*</td>
</tr>
<tr>
<td>Doxorubicin 18 mg/kg</td>
<td>507.3±17.38*#</td>
<td>73.2±3.2*#</td>
<td>1.93±0.149**#</td>
<td>1.25±0.121**#</td>
</tr>
<tr>
<td>Doxorubicin 18 mg/kg + L Carnitine</td>
<td>223±35.8*#</td>
<td>49.5±2.014*#</td>
<td>4.02±0.165*#</td>
<td>2.42±0.144*#</td>
</tr>
</tbody>
</table>

Table 4. The effect of doxorubicin on the activity of antioxidant enzymes, glutathione peroxidase (GSHPx), Catalase (CAT), glutathione reductase (GR) and glutathione-s-transferase (GST), in Rat liver tissues

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>GSHPx µmol/g wet tissue</th>
<th>CAT µmol/min/g tissue</th>
<th>GR ng/ml</th>
<th>GST µmol/min/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>117.5±3.308</td>
<td>67.5±1.08</td>
<td>0.953±0.02</td>
<td>34.2±1.398</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>118.3±2.983</td>
<td>70.80±0.789*</td>
<td>0.855±0.008*</td>
<td>34.5±1.08</td>
</tr>
<tr>
<td>Doxorubicin 6mg/kg</td>
<td>83.5±2.068*</td>
<td>35.4±0.699*</td>
<td>0.534±0.05*</td>
<td>21.8±1.033*</td>
</tr>
<tr>
<td>Doxorubicin 12 mg/kg</td>
<td>51.5±1.509*#</td>
<td>37.4±1.265*#</td>
<td>0.425±0.006*</td>
<td>22.0±1.33*#</td>
</tr>
<tr>
<td>Doxorubicin 18 mg/kg</td>
<td>42.6±1.578*#</td>
<td>26.8±1.030*#</td>
<td>0.27±0.11*#</td>
<td>18.1±0.994*#</td>
</tr>
<tr>
<td>Doxorubicin 18 mg/kg + L Carnitine</td>
<td>106.1±3.510*#</td>
<td>66.3±0.472*#</td>
<td>1.01±0.046*#</td>
<td>36.2±1.398*#</td>
</tr>
</tbody>
</table>

Table 5. Effect of doxorubicin on the gene expression level of the antioxidant enzymes, glutathione peroxidase (GSHPx), Catalase (CAT), glutathione reductase (GR) and glutathione-s-transferase (GST), in Rat liver tissues

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>GSHPx</th>
<th>CAT</th>
<th>GR</th>
<th>GST</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Carnitine</td>
<td>1.9 ±0.288*</td>
<td>1.826±0.348*</td>
<td>2.268±0.273*</td>
<td>2.972±0.338*</td>
</tr>
<tr>
<td>Doxorubicin 6mg/kg</td>
<td>0.957±0.116</td>
<td>0.65±0.126</td>
<td>0.659±0.0128*</td>
<td>1.022±0.147</td>
</tr>
<tr>
<td>Doxorubicin 12 mg/kg</td>
<td>0.588±0.115</td>
<td>0.415±0.064*</td>
<td>0.5428±0.118*</td>
<td>0.688±0.107</td>
</tr>
<tr>
<td>Doxorubicin 18 mg/kg</td>
<td>0.376±0.115</td>
<td>0.213±0.047*</td>
<td>0.373±0.052*</td>
<td>0.583±0.066* #</td>
</tr>
<tr>
<td>Doxorubicin 18 mg/kg + L Carnitine</td>
<td>42.6±1.578*#</td>
<td>26.8±1.030*#</td>
<td>0.27±0.11*#</td>
<td>18.1±0.994*#</td>
</tr>
</tbody>
</table>

Discussion

Doxorubicin is potent anticancer drug, whose clinical use is limited on account of its toxicity. It causes disruption in basal metabolism by showing toxic effect especially in liver and heart tissues in animals. It is known that doxorubicin is considered the most toxic anthracyclines and causes weight loss for this reason, the long term and over dosage of this drug causes death. Since oxidation stress is considered to be the major event responsible for anthracyclines toxicity. During our experiments we used suitable Dox doses because there was no record for any animal death. The current study investigates whether L-carnitine supplementation could inhibit the hepatotoxicity induced by cumulative dose of doxorubicin in rats.

Data presented in this study demonstrate that doxorubicin increased serum indices of liver function including ALT, ALP and total bilirubin. These elevations of ALT and ALP are attributed to hepatocellular damage and decreased liver functions. These elevated levels of serum indices for hepatocellular damage has been previously reported in Dox-induced hepatotoxicity model. Interestingly, L-carnitine prevents the increase in hepatic enzymes during its administration in combination with a cumulative dose of doxorubicin. These results suggested that L-carnitine may have protective effect against Dox-induced liver damage. This protective effect might be due to stabilization of hepatocyte membranes by L-carnitine with the consequent decrease in the leakage of liver enzymes.
and his colleagues\(^{37}\) which showed that biosynthesis of carnitine is decreased in pediatric patients receiving valproic acid. The level of carnitine in hepatocytes is controlled by the specific carnitine transporter (OCTN-2) and endogenous synthesis.\(^{38,39}\) Decreased expression of OCTN-2 has been reported in the acute hepatitis.\(^ {40}\) Also, OCTN-2 located on hepatocyte membranes might be destroyed when exposed to ROS induced by doxorubicin.

Damage, at the cellular level by oxidants, is attenuated by antioxidant enzyme such as SOD, GSHPx, GSP, CAT and GR.\(^ {41}\) Superoxide dismutate is one of the major enzymatic antioxidant mechanisms against superoxide radical, prevents liver toxicity induced by Dox.\(^ {2}\) Catalase and GSHPx catalyze dismutation of the superoxide anion (\(O_2^-\)) into hydrogen peroxide (\(H_2O_2\)) which then converting \(H_2O_2\) to water thus providing protection against reactive oxygen species.\(^ {42}\) The reduction in activity of these enzymes may be caused by the increase in free radical production during doxorubicin metabolism.\(^ {32,43}\) Our study showed that cumulative dose of doxorubicin significantly increase both the gene expression level of NOx and TBARS in liver tissue and their enzymatic level in serum. Our study also showed decrease in the gene expression levels of GSHPx, CAT, GR and GST in liver tissue with the cumulative dose of doxorubicin with decrease in their activity in the serum. These data showed that doxorubicin not only increase the free radical formation but also decrease its ability to detoxify reactive oxygen species. The formation of superoxide radicals together with NO might form peroxynitrite induced by doxorubicin causes tissue damage leading to an increase in the levels of TBARS and NOx. This was in agreement with several other reports.\(^ {7,44}\) In contrast to our study, Kalender and his colleagues\(^ {45}\) found that administration of doxorubicin (5 mg/week for 6 weeks) increased the levels of TBARS and SOD, and the activity of both GSHPx and catalase enzymes. This difference may be due to the difference in the study design between our study and their.

Antioxidants agent like L-carnitine have been found to offer protection against Dox-induced liver damage.\(^ {36}\) It was used to prevent the toxic effect of cisplatin-induced nephrotoxicity by normalized kidney function, where oxidative stress and lipid peroxidation play a major role in this toxicity. In addition, L-carnitine attenuated the increased TBARS and reduced GSH levels,\(^ {46}\) in which there were no toxic effects of it on approved doses. Data from this study revealed that L-carnitine administration in combination with doxorubicin significantly increase antioxidant enzyme and decrease the indexes of the lipid peroxidation. The increase in GSH level lead to increase in the activity of GSHPx in which the former acts as a cofactor for later.\(^ {48}\) Our results are consistent with previous studies reported that L-carnitine had similar non-enzymatic free-radical scavenging and anti-lipid peroxidation activities.\(^ {36,49}\)

**Materials and Methods**

**Animals.** Adult male Wistar albino rats, weighing 230–250 g, were obtained from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were housed in metabolic cages under controlled environmental conditions (25°C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet diet essentially carnitine free and tap water unless otherwise indicated. The protocol of this study has been approved by Research Ethics Committee of College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

**Materials**

Doxorubicin was a generous gift from King Khaled University Hospital drug store. L-carnitine was kindly supplied by Dr. Zaven Orfalian, Sigma-Tau Pharmaceuticals, Pomezia, Italy. It has been supplied as white powder in a non-commercial plastic bottle contains 100 g and it was freshly dissolved in normal saline prior to injection. All other chemicals used were of the highest analytical grade.

**Experimental design and doxorubicin treatment protocol.** In this study, the doxorubicin treatment regimen (3 mg/kg every other day) used to develop the cumulative hepatotoxicity was adopted from our previous study\(^1\) with slight modification in which animals were sacrificed 24 hours after cumulative doses of 6, 12 and 18 mg/kg. To achieve the ultimate goal of this study, a total of 60 adult male Wistar albino rats were used and divided at random into 6 groups of 10 animals each. Group one was injected intraperitoneally (i.p.) with normal saline (2.5 ml/kg) and served as a normal control. Group two was injected, every other day, with doxorubicin (3 mg/kg, i.p.) over a period of 3 days to obtain cumulative dose of 6 mg/kg. Group three was injected, every other day, with doxorubicin (3 mg/kg, i.p.) over a period of 7 days to obtain cumulative dose of 12 mg/kg. Animals in the fourth group were injected, every other day, with doxorubicin (3 mg/kg, i.p.) over a period of 11 days to obtain cumulative dose of 18 mg/kg. Group five was injected with L-carnitine (200 mg/kg, i.p.) for 10 consecutive days. Animals in the sixth group were received doxorubicin (18 mg/kg) as group 4 and L-carnitine (200 mg/kg) as group 5. At 24 hours after receiving the last dose of doxorubicin, animals were anesthetized with ether and blood samples were obtained from the retro-orbital sinus of the eye. Sera were separated for measurement of alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin and total nitrate/nitrite (NOx). Immediately after blood samples were collected, animals were then sacrificed by decapitation after exposure to ether in a desiccators kept in a well-functioning hood and their livers were rapidly excised, weighed, washed with saline, blotted with a piece of filter paper and homogenized in normal saline to yield a 10% (w/v) tissue homogenate, using a Branson sonifier (250 VWR Scientific, Danbury, CT). Liver specimens from each group were removed and kept in -80°C until used.

**Methods**

Detection of gene expression level by real time PCR in liver tissues.

**Total RNA extraction.** Total RNA were extracted from liver tissue by Trizol method according to the manufacturer’s protocol as previously described in reference 50. The quantity and
integrity were characterized using a UV spectrophotometer. RNA was electrophoresed on ethidium bromide stained agarose gel. The isolated RNA has an A 260/280 ratio of 1.9–2.1.

cDNA synthesis and real time PCR methods. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription with a SuperScript™ first-strand synthesis system kit (Invitrogen, CA USA), according to the manufacturer’s instructions. Real time PCR was done according to our previous study in reference 1. We used GAPDH gene as housekeeping gene. All primers were listed in Table 1. Following amplification, melting curve analysis was performed to verify the correct product according to its specific melting temperature (Tm).

Determination of free and total carnitine in serum and liver tissues. Liver homogenate was prepared in ice-cold 6% perchloric acid and centrifuged at 8,000 g for 10 min. Part of the supernatant was used for the estimation of free carnitine, while the remainder was used for the determination of long-chain acyl carnitine after hydrolysis in KOH 1 mol/L at 65°C for 1 h as previously described in reference 51. Carnitine was determined using HPLC after pre-column derivatization with L-aminoantracene as previously described in reference 52.

The mobile phase was prepared by mixing 700 ml of 0.1 mol/L ammonium acetate, pH 3.5, with 300 ml of acetonitrile. Chromatographic separation was performed at a flow rate of 1.5 ml/min, using a Kromasil column (C18, 25 cm x 4.6 mm) from Saulenttechnik Knayer, Berlin, Germany. The excitation and emission wavelengths of the spectrofluorimeter were 248 and 418 nm, respectively.

Determination of glutathione peroxidase, catalase, glutathione transferase, glutathione reductase activity, total nitrate/nitrite and superoxide dismutase. Determination of the activity of glutathione peroxidase (GSHPx) was done as previously described in reference 53. The changes in the absorbance at 340 nm were recorded at 1 min interval for 5 min and the results were expressed as μmol/min/g tissue. The catalase (CAT) activity was determined spectrophotometrically by the method of Pljesa-Ercegovac et al. with slight modification, the activity was expressed as μmol/min/g tissue using the molar absorbance of 43.6 for hydrogen peroxide. Glutathione transferase activity was assayed as previously described in reference 55. Using 1-chloro-2,4-dinitrobenzene and the results were expressed as nmol/min/mg protein. The glutathione reductase was assayed by ELISA kit (Uscn Life Science Inc., Wuhan) based on sandwich enzyme immunoassay. The activity was expressed as ng/ml. The nitrite and nitrate (NO) levels were determined according to the previous described method based on the Griess reaction. Samples were initially deproteinized with Somogyi reagent. Total nitrite was measured by spectrophotometry at 545 nm after the conversion of NO2- to NO3- by copperized cadmium granules. Results were expressed as micromole per gram tissue protein (μmol/g protein). Tissue SOD activity was determined using the nitroblue tetrazolium (NBT) method described by Sun et al. and modified by Durak et al.

In this method, NBT is reduced to blue formazan by superoxide (O2•−), which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. SOD activity was expressed as units per mg tissue protein (U/mg protein).

Statistical analysis. Differences between obtained values (mean ± SEM, n = 10) were carried out by one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A p-value of 0.05 or less was taken as a criterion for a statistically significant difference.

Conclusion

In conclusion, the present study showed that L-carnitine deficiency is a risk factor and should be viewed as a mechanism in Dox-induced liver damage. The cumulative dose of doxorubicin causes hepatic toxicity as a result of reactive oxygen species may be prevented by L-carnitine supplementation. Therefore, L-carnitine supplementation during Dox treatment may be used to prevent, slow or reverse the occurrence of liver damage.

Acknowledgements

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References
