Ameliorating effect of DL-α-lipoic acid against cisplatin-induced nephrotoxicity and cardiotoxicity in experimental animals

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ABSTRACT: Cisplatin is a potent chemotherapeutic agent with a wide range of activities. Nephrotoxicity and cardiotoxicity represent its major complication upon clinical use. The present study was carried out to evaluate the possible protective effect of DL-α-lipoic acid (LA) against cisplatin-induced nephrotoxicity and cardiotoxicity. Different groups of rats (n = 10) were administered either saline (control), cisplatin (10 mg/kg, i.p.), LA (100 mg/kg, i.p.) or their combination (LA 30 min prior to cisplatin administration). Twenty-four hours later all animals were decapitated and sera were used for estimation of activities of urea (BUN), creatinine (Cr), lactate dehydrogenase (LDH), and creatine kinase (CK). Homogenates of the kidney and heart were used for estimation of oxidative stress markers (reduced glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and nitric oxide (NO)). Additionally, caspase-3 activities and DNA-fragmentation were investigated in renal tissues. The results showed that cisplatin produced significant elevation in serum activities of LDH, CK, BUN, and Cr and also induced significant elevation in the oxidative stress makers (MDA and NO) accompanied by significant reduction in GSH and SOD in both kidney and heart. The integrity of DNA was heavily damaged and caspase-3 was activated in renal tissues. The results emphasized nephrotoxicity and cardiotoxicity of cisplatin. On the other hand, prior administration of LA significantly attenuated the cisplatin-evoked disturbances in the above mentioned parameters and protected both kidney and heart tissues. The histopathological examination emphasized the obtained results. In conclusion, LA is suggested to be a potential candidate to ameliorate cisplatin-induced nephrotoxicity and cardiotoxicity without altering the antitumor efficacy of cisplatin.

Keywords: Cisplatin, DL-α-lipoic acid, oxidative stress, nephrotoxicity, cardiotoxicity

1. Introduction

Cisplatin is a potent chemotherapeutic agent that has wide range of activity against different tumors such as testicular, head and neck, ovarian, and non-small cell lung cancers as well as hematological malignancies (1). Despite its usefulness, major side effects such as nephrotoxicity, cardiotoxicity, neurotoxicity, emetogenesis, and ototoxicity have limited its use in clinical treatment (2). Several studies have documented the involvement of generation of reactive oxygen species, such as superoxide anion and hydroxyl radical in its mechanism of toxicity (1,3) with reduction of various antioxidants in patient plasma (4). Failure of the antioxidant defense mechanism against free radical-mediated organ damage after cisplatin administration was also recorded (5). All these mechanisms greatly encourage the using of free radical scavengers and antioxidants to counteract cisplatin-induced toxicities (6,7).

DL-α-Lipoic acid (LA), a dithiol compound, is found naturally in the mitochondria and acts as an essential cofactor for mitochondrial respiratory enzymes pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. It displays antioxidant effects by scavenging reactive oxygen species and stimulates the synthesis of other antioxidants, such as glutathione (8). Several studies have shown that LA exerts multiple pharmacological actions in different models of diseases characterized by increase in oxidative stress markers (9-11). Additionally, the cardioprotective effects of LA against Adriamycin and cyclophosphamide induced-toxicities are well documented (12,13). Moreover, LA exerts anti-inflammatory actions by inhibiting nuclear factor-κB (NF-κB) activation and by decreasing adhesion molecule expression in endothelial cells (14). Accordingly, in the present investigation, LA was suggested to be a good candidate for protection against cardiotoxicity and nephrotoxicity induced by cisplatin.
The aim of the present study was to evaluate the possible efficacy of LA as a protective agent against cisplatin-induced nephrotoxicity and cardiotoxicity in rats and to investigate whether this protection may affect the antitumor activity of cisplatin.

2. Materials and Methods

2.1. Chemicals and drugs

Cisplatin vials (Bristol Myers Squibb Co., USA) were used. DL-α-lipoic acid was purchased from Sigma-Aldrich Chemie, Germany. All other chemicals and reagents used were of the highest purity grade available and were purchased from Sigma-Aldrich Chemie, Germany.

2.2. Animals

Adult male Wistar albino rats weighing 180-200 g purchased from the Egyptian Organization for Biological Products and Vaccines (VACSERA, Giza, Egypt) and Female Swiss albino mice weighing 22-25 g obtained from animal house facility, Pharmacology Unit, National Cancer Institute (NCI), Cairo University, Egypt, were used in this study. Animals were kept under standardized conditions (temperature 22 ± 1ºC, relative humidity 55 ± 15% with a 12-h light and dark cycle and were allowed food and tap water ad libitum). The animal's treatment protocol has been approved by the animal care committee of the National Cancer Institute, Cairo University, Egypt. The protocol is in accordance with the international guidelines of handling the experimental animals.

2.3. Experimental design

The doses of cisplatin and LA were chosen according to a pilot study and were matched with that in the literature (15). The current study includes two parts: i) estimation of the effect of LA on cisplatin producing reduction in tumor volume in mice bearing solid Ehrlich carcinoma (SEC); ii) evaluation of the protective effect of LA against cisplatin-induced toxicities in kidney and heart of rats.

In the first part, solid tumor was transplanted subcutaneously in the right thigh of the lower limb of each mouse. Mice with a palpable tumor mass (100 mm³) that developed within 7 days after implantation were divided into 4 groups (n = 10) and followed the same treatment in previous experimental design. The change in tumor volume was measured three times weekly using a vernier caliper and calculated by the following formulas previously described (16).

\[
\text{Tumor volume} = \frac{4\pi (A/2)^2 \times (B/2)}{3} = 0.52 \times A^2 \times B, \text{ where } A \text{ and } B \text{ denoted the minor and major tumor axis, respectively.}
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In the second part, rats were divided into four groups (n = 10). In one group, rats were injected with 0.2 mL saline solution and served as control group. In the second group rats were injected with single dose of cisplatin (10 mg/kg, i.p.). In the third group, rats were administrated LA (100 mg/kg, i.p.). In the fourth group, rats were administrated LA (100 mg/kg, i.p.) followed by cisplatin (10 mg/kg, i.p.) 30 min later. Twenty-four hours after the last dose of the specific treatment, all animals were sacrificed by decapitation under light ether anesthesia. Blood samples were collected from heart puncture and serum samples were separated for measurement of renal function tests and serum cardiac enzymes. The kidney and heart were excised, immediately washed in ice-cold isotonic saline, blotted between two filter papers and used for preparing homogenates (20%, w/v) with normal saline, and were kept at −20°C till estimation of oxidative stress markers. Parts of the kidney homogenate were used for evaluation of DNA fragmentation and caspase-3 activation. Samples of the intact tissue of heart and kidney were washed with saline and fixed in 10% neutral buffered formalin solution for histopathological examination.

2.4. Determination of biochemical parameters

2.4.1. Cardiac biomarkers

Serum cardiac enzymes lactate dehydrogenase (LDH) and creatine kinase (CK) were determined using standard commercial kits (Spectrum diagnostics, Cairo, Egypt) according to the methods previously described by Bais and Philcox (17), and Wu and Bowers (18), respectively. The values were expressed as U/L and were presented as percent of control values.

2.4.2. Renal biomarkers

Serum levels of urea (BUN) and creatinine (Cr) were determined using standard commercial kits (Spectrum diagnostics, Cairo, Egypt) and according to the methods described by Tabacco et al. (19), and Bowers and Wong (20), respectively. The values were expressed as mg/dL and were presented as percent of control values.

2.5. Determination of oxidative stress markers

2.5.1. Estimation of tissue lipid peroxidation contents

MDA is formed from the breakdown of polyunsaturated fatty acids, and serves as a convenient index for determining the extent of the peroxidation reaction. MDA was measured according to the method of Buege and Aust (21). The results were expressed as nmol/g tissue.

2.5.2. Measurement of tissue glutathione (GSH) contents

Reduced GSH was measured spectrophotometrically at 412 nm according to the method of Ellman (22). The amount of GSH was expressed as µmol/g tissue.

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2.5.3. Determination of tissue superoxide dismutase (SOD) activity

SOD activity was determined spectrophotometrically at 540 nm following the inhibition of pyrogallol autooxidation as described previously by Minami and Yoshikawa (23). The rate of inhibition of pyrogallol autooxidation is directly proportional to the activity of SOD in the tissues. The values of SOD were expressed as μmol/mg tissue.

2.5.4. Assessment of tissue total nitrate/nitrite (NO) concentration

Total NO was measured chemically according to the method of Miranda et al. (24). The levels of NO were expressed as μmol/g tissue in homogenate.

2.6. Evaluation of DNA fragmentation in the kidney tissues

DNA fragmentation in the kidney was evaluated according to the method described by Katoh et al. (25) using agarose gel electrophoresis. The kidney tissues were homogenized and lysed in a cold lysis buffer (10 mM Tris-HCl, 5 mM disodium EDTA, and 0.5% Triton X-100, pH 8.0) for 10 min at 4°C. The DNA was sequentially extracted twice using half volumes of phenol/chloroform and incubated at 55°C for 10 min. After centrifugation at 3,000 rpm for 20 min, the upper layer was incubated with proteinase at 37°C for 60 min followed by incubation with ribonuclease at 37°C for 60 min. The DNA was precipitated by adding 10 M ammonium acetate and 100% ethanol and maintained at −20°C overnight. DNA was collected by centrifugation at 15,000 × g for 20 min, air-dried, and resuspended in TBE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.4). The resulting DNA preparations were electrophoresed through a 1.4% agarose gel containing ethidium bromide using TBE buffer (Tris-boric acid-EDTA buffer, pH 8.3) at 40 V for 5 h. Equal quantities of DNA (based on optical density measurements at 260 nm) were loaded in each lane, and a molecular DNA marker was used as a molecular mass standard (26). DNA fragmentation was visualized and photographed under ultraviolet illumination for testing the degree of fragmentation.

2.7. Evaluation of caspase-3 activities in the kidney tissues

The kidney tissues were homogenized followed by centrifugation at 11,000 rpm, 4°C for 15 min. Protein content in the resulting supernatant was determined using Bradford reagent (Thermo scientific, USA). Equal volume of supernatant was mixed with 1× loading buffer and 5 μL of β-mercaptoethanol and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and then electrotransferred onto PVDF membrane using semidyry transfer apparatus. The membrane was blocked according to manufacture instruction of chromogenic western max detection kit (Ameresco, USA). After blocking with dilution buffer DBT (DB plus 1% Tween-20) plus 1% bovine serum albumin (BSA), the membrane blots was incubated within dicated primary antibody at 4°C over night then washed, and incubated with horseradish peroxidase-conjugated secondary antibody for 4 h at room temperature. After washing, the protein bands were visualized by 3,3-diaminobenzidine (DAB) chromogen which gives brown precipitate at the reaction site, specific protein bands on these transferred membranes were detected using antibodies (purified anti-β-actin antibody obtained from (Biolegend, USA) and anti-mouse caspase 3 monoclonal antibody obtained from (Bioscience, USA)). Relative expression of proteins was evaluated by normalizing the expression of proteins with quantitative housekeeping protein β-actin (27).

2.8. Histopathological examination

Heart and kidney samples were kept in 10% neutral buffered formalin and were dehydrated through alcohols, cleared in xylene and then embedded in paraffin wax. Sections (5 mm thick) were stained with haematoxylin and eosin. The magnification power of 400 was used to elucidate the histopathological changes using light microscopy.

2.9. Statistical analysis

The results were expressed as (mean ± SEM). The comparison between means of the groups (n = 10) were carried out using one way analysis of variance (ANOVA) followed by the Tukey multiple comparison test. p value of 0.05 or less was taken as a criterion for a statistically significant difference.

3. Results

3.1. Effect of cisplatin, DL-α-lipoic acid and their combination on tumor volume

As shown in Table 1, administration of a single dose of cisplatin resulted in a significant decrease in the tumor volume (95.57 ± 16.44 mm³) as compared to the control group. Treatment with LA (100 mg/kg) alone
did not produce any significant change in the tumor volume (268.65 ± 103.34 mm$^3$) as compared to control. Administration of LA (100 mg/kg) 30 min prior to cisplatin produced a significant decrease in the tumor volume (98.25 ± 14.39 mm$^3$) as compared to control. However this effect was not significant from cisplatin group.

3.2. Biomarkers of cardiotoxicity

Results of the current study revealed that administration of cisplatin (10 mg/kg) resulted in a significant increase in serum LDH and CK levels to 33% and 140% compared to the control group, respectively. This indicates the marked heart injury. In contrast, co-administration of LA with cisplatin induced a significant reduction in serum LDH and CK compared to cisplatin group (Figures 1A and 1B).

3.3. Biomarkers of nephrotoxicity

As shown in Figures 2A and 2B, treatment with a single dose of cisplatin (10 mg/kg) resulted in a significant increase in BUN (155%) and the Cr (219%) compared to the control. Administration of LA (100 mg/kg) resulted in a non significant change in BUN or Cr levels, compared to the control group. Meanwhile, co-administration of LA (100 mg/kg) 30 min before cisplatin (10 mg/kg) resulted in a complete reversal of cisplatin-induced increase in BUN or Cr levels compared to the cisplatin values.

3.4. Biochemical markers of oxidative stress

3.4.1. Effect on lipid peroxidation level

Results in Tables 2 and 3 showed that, cisplatin administration to rats significantly increased the MDA
levels in both heart and kidney tissues, by 62% and 90%, respectively, compared to the control group. Administration of LA with cisplatin diminished the cisplatin-induced increase in MDA levels in both tissues. Moreover, LA normalized the MDA in these tissues.

3.4.2. Effect on reduced GSH content

LA produced a significant elevation in the levels of reduced GSH in the heart and kidney tissues. On the other hand, cisplatin administration, significantly decreased the GSH levels in the heart and kidney tissues by 46% and 61%, respectively, compared to the control (Tables 2 and 3). Administration of LA (100 mg/kg) 30 min before cisplatin (10 mg/kg) resulted in a significant decrease in heart tissues content of nitric oxide (43%) as compared to the control, and a significant decrease (65%) as compared to cisplatin group. Co-administration of LA (100 mg/kg) with cisplatin (10 mg/kg) resulted in a significant decrease in kidney tissues content of nitric oxide (42%) as compared to the control, and a significant decrease (60%) as compared to cisplatin group.

3.5. Evaluation of DNA fragmentation in the kidney tissue

As illustrated in Figure 3, no appreciable fragmentation of DNA was observed in the kidney tissues of control rats, LA, and the combination group. Whereas, cisplatin exposure resulted in a marked damage of DNA in the kidney tissues.

3.6. Evaluation of caspase-3 activities in the kidney tissue

The results in Figure 4 revealed that, administration of cisplatin induced a significant increase in cleaved caspase-3 in the kidney tissues. Treatment with LA 30 min before cisplatin administration showed a significant decrease in cleaved caspase-3 in the kidney compared with the cisplatin-treated group.

3.7. Histopathological examination

Figures 5A and 5B indicated that, LA prevented cisplatin-induced kidney and heart damage. Where
24 h after cisplatin treatment, extensive renal tubular injury were observed, which included tubular cell necrosis, extensive degeneration, and sever damage in some proximal and distal tubules with loss of normal architecture of cells. Moreover, interstitial hemorrhage between the kidney tubules was also observed (Figure 5A). The heart section of cisplatin treatment showed separated cardiac muscles with interruption of myofibrils and severs interstitial hemorrhages (Figure 5B). Cisplatin administration significantly increased kidney and heart injury compared with the control group. Treatment with LA significantly reduced the cisplatin-induced kidney and heart damage. Treatment with LA alone caused no significant morphologic alterations in both tissues.

4. Discussion

In the current study, administration of cisplatin produced a significant decrease in the tumor volume as compared to control group indicating its powerful chemotherapeutic activity. LA alone showed no effect on tumor volume indicating that it has no antitumor activity. Additionally, co-administration of LA with cisplatin produced a non-significant change in the antitumor activity of the latter, indicating no change in the efficacy of cisplatin upon combination with LA.

In the present study, cisplatin significantly increased cardiac enzymes (CK, LDH) activities. A significant increase was also observed in kidney...
function tests (BUN, Cr) compared with control group. Previous studies reported similar elevation in CK and LDH after cisplatin administration (15,28,29). The elevation in activities of these enzymes in serum is diagnostic marker for cardiac injury. Cisplatin has the ability to generate reactive oxygen species, such as superoxide anion and hydroxyl radical (3), that results in irreversible modification of myocardial membrane structures, functions and integrity with the consequent leakage of cardiac enzymes (13,15).

Nephrotoxicity is one of the most common side effects of cisplatin encountered in cancer patients. More than 25% of patients developed acute renal failure after receiving an initial dose of cisplatin due to its preferential accumulation within the proximal tubular epithelial cells (30-32). Nephrotoxicity was manifested by significant increase in urea and creatinine resulted from reduction of glomerular filtration rate (30-33). Several factors including inflammation, genotoxic damage, and cell cycle arrest have been incriminated in the pathogenesis of cisplatin nephrotoxicity (34,35).

The marked oxidative stress, as evidenced by increased lipid peroxidation and decreased antioxidant capacity in heart and kidney tissues, observed in the current study after cisplatin treatment is in accordance with previous reports (15,34,35). This decline in antioxidants further aggravates the levels of free radicals in heart and kidney and prevents organ protection against cisplatin toxicity (3,15,36,37).

In one study, the low cardiac glutathione level was a risk factor for developing cyclophosphamide-induced congestive heart failure (38). The heart and kidney have a variety of endogenous enzymatic and non-enzymatic antioxidants that act in coordination to provide cellular defense against reactive oxygen species.

In the present work, nitric oxide levels in the heart and kidney were significantly elevated following cisplatin administration. Overproduction of nitric oxide was directly linked to heart damage in other models of chemotherapeutic agents-induced cardiotoxicity, as in cyclophosphamide (13), and doxorubicin (39). Negative inotropic effects with deterioration of myocardial performance and the induction of myocardial damage (40) were attributed to overproduction of NO. In addition, it has been demonstrated that NO may enhance cellular injury by decreasing intracellular GSH levels (41). Nitric oxide promotes oxidative stress-induced cell injury by formation of peroxynitrite anion, a potent prooxidant and cytotoxic intermediate, that causes protein nitration and tissue injury (42,43).

Another possible explanation may be attributed to the fact that cisplatin induces a cascade of inflammatory reactions, which play an important pathogenic role in cisplatin-induced tissues injury (2).

The activation in caspase-3 accompanied with DNA fragmentation in kidney tissues of cisplatin treated rats observed in the present study can be explained on the bases that, the reactive oxygen species generated by cisplatin may trigger the opening of the mitochondrial permeability transition pores and permits the release of cytochrome c from mitochondria to cytosol and hence activates the mitochondria dependent pathway leading to apoptosis (44). Caspase activation is thought to be important in the genesis of apoptosis, in particular, caspase-3, the execution caspase, which is instrumental in the apoptotic process, and it cleaves and activates poly (ADP-ribose) polymerase and the inhibitor of caspase activator domain protein, leading to DNA fragmentation (45,46).

In the present study, administration of LA alone, increased the level of GSH, compared to the control group, a finding which is in agreement with that of Melhem et al. (47). This may be explained on the bases that LA and their reduced form (dihydrolipoic acid) may act as extra- and intracellular redox couples and powerful lipophilic free radical scavenger (48). It is well documented that, LA is a powerful free radicals scavenger and capable of increasing concentration of GSH in tissues (49). Additionally, LA is involved in recycling of antioxidant vitamins C and E and in modulating the activities of SOD (50). There is mounting evidence that LA increases or maintains cellular GSH levels by acting as a transcriptional inducer of genes governing GSH synthesis (51).

In the present study the beneficial effects of LA were manifested by significant decrease in serum levels of cardiac enzymes (LDH and CK) and in kidney biomarker enzymes (BUN and Cr). Additionally, MDA levels in heart and kidney tissues were significantly diminished and the antioxidants GSH level and SOD activity were restored. Similar decrease in cardiac enzymes activities by LA in rats treated with doxorubicin (52) as well as in rats treated with cisplatin (15) was previously reported. Moreover, LA was effective in normalizing the antioxidant levels, as well as levels of creatinine and blood urea nitrogen in acetaminophen-induced renal damage in rats (36).

In another study, LA attenuated the elevation in creatinine and blood urea nitrogen induced by cisplatin in mice (2). Additionally, comparable effects of LA on the antioxidant defense system and prevention of the increase in lipid peroxidation were previously reported in renal tissues (53) and in cochlear tissues (54) of rats treated with cisplatin. LA has strong ability to chelate metals and to scavenge free radicals such as hydroxyl radical (15,55). In addition, LA is easily absorbed and transported across cell membranes, thus, free radical protection occurs both inside and outside of cells (56). LA not only exerts potent antioxidant activities but also has anti-inflammatory effects against different inflammatory conditions (2,11,37).
There is an ample of evidence indicating that inhibition of nitric oxide overproduction by LA can protect against the heart and kidney injury induced by other chemicals or drugs (39,41). The ability of LA to modify nitric oxide production by preventing the up-regulation of nitric oxide synthase was previously documented, where LA inhibits lipopolysaccharide-induced NO production in isolated rat Kupffer’s cells and in murine macrophages (58). Furthermore, pretreatment with LA may protect against cisplatin-induced kidney and heart injury through prevention of NO overproduction (36,59). Another possible explanation is the direct scavenging effect of NO by the sulphydryl group of LA (60).

In the current investigation, the significant decrease in cleaved caspase-3 and DNA fragmentation induced by cisplatin observed after LA treatment can be explained by the protective and the anti-apoptotic properties of LA. This protective effect may be attributed to its antioxidant action exerted against the pro-oxidant effects of cisplatin. LA is a hydroxyl radical scavenging agent. It prevents hydroxylation of deoxyguanosine and thus the associated DNA damage (61). Furthermore, LA inhibited the site-specific degradation of deoxyribose by prooxidants due to its iron chelating ability (62).

In histopathological examination, the observed abnormalities in kidney tissues observed after cisplatin treatment are in accordance with the previous studies (31,63,64). As cisplatin-induced nephrotoxicity exhibits histological alterations including swelling and vacuolation of the lining endothelium of the glomerulus tuft as well as tubular degeneration of the renal tubular cells. These changes are associated with the loss of renal functions as revealed by the observed abnormalities in renal enzymes. On the other hand, the concurrent administration of LA with cisplatin, almost prevents the histopathology changes of the heart and kidney, especially necrosis and sever interstitial hemorrhages (2).

5. Conclusion

It could be concluded that, cisplatin administration results in pronounced oxidative stress which is revealed by cellular damage to the heart and kidney of the rats. Co-administration of dl-α-lipoic acid was found to be effective candidate in protecting the heart and kidney tissues from cisplatin-induced nephrotoxicity and cardiotoxicity without interfering with antitumor activity of cisplatin. It is greatly recommended to co-administer dl-α-lipoic acid with cisplatin to minimize the nephrotoxicity and cardiotoxicity of the latter. Further clinical studies are encouraged to approve the validity of these results in human.

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