Protective Effect Of L-Carnitine Against Amiodarone-Induced Lung Toxicity In Rats
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Abstract
Amiodarone (AMD) is a potent antiarrhythmic drug that is limited in clinical use by its potentially life-threatening AMD-induced lung toxicity. The resemblance of the morphologic changes of lungs in AMD-treated rats to pulmonary toxicity in humans, suggest that this type of chemical injury may serve as useful model of this disease entity. The effect of L-Carnitine (CAR) on AMD-induced lung toxicity was investigated in rats. AMD induced lung toxicity, manifested biochemically by a significant decrease in reduced glutathione contents, catalase and superoxide dismutase enzymes activities in lung tissues.  Also, AMD induced a significant increase in lipid peroxides, measured as malon-di-aldehyde (MDA) and nitric oxide (Nitrite -Nitrate) in lung tissues. Moreover, AMD induced pulmonary toxicity was further confirmed by histopathological findings. Interestingly, administration of L-carnitine to AMD treated rats completely reversed the biochemical and histopathological changes induced by AMD to the control values. These results suggest that CAR can ameliorate lung dysfunction induced by MDA via a mechanism(s) which involves the production of nitric oxide rather than lipid peroxidation. In addition, CAR may therefore be a beneficial remedy for AMD lung toxicity and can be used to improve the therapeutic index of AMD

INTRODUCTION
Amiodarone (AMD), an iodinated benzofuran derivative, is a very effective class III antiarrhythmic drug that inhibits ventricular fibrillation and ventricular tachyarrhythmia [1, 2]. However the use of amiodarone is often limited by adverse reactions involving many different organ systems. Pulmonary toxicity is one of the most life threatening complications of amiodarone use. Amiodarone induced pulmonary toxicity occurs with the incidence of 5–10% and it is fatal in considerable number of patients [3–5]. The incidence of pulmonary toxicity depends on the dosage and duration of amiodarone used.

Many studies have shown an increased risk of pulmonary toxicity in patients receiving amiodarone when the dosage exceeds 400 mg/day administered for more than 2 months or when a lower dosage is given for more than 2 years [6–8]. On the other hand, Ott et al. [9] have also reported that 200 mg/ day of amiodarone can increase the risk for pulmonary toxicity. Unfortunately, there has been few effective prevention or cure for amiodarone-induced pulmonary toxicity, except for the immediate withdrawal of the compound and the prompt corticosteroid therapy [8, 9].

The etiology of pulmonary toxicity caused by amiodarone remains to be clarified, emerging evidence that amiodarone administration results in interstitial alveolar inflammation and reports from earlier studies mentioned that coagulation abnormalities with oxidative metabolic reactions in lung injury are compelling reasons to use of antioxidant such as soybean in suppressing amiodarone induced pulmonary toxicity [10]. Several studies suggest that oxidant-antioxidant imbalances in the lower respiratory tract play a critical role in the pathogenesis of pulmonary injury. For example, pulmonary inflammatory cells of patients with pulmonary injury generate higher levels of oxidants than those in control patients [11]. Therefore, in the present study, we focused on the possible effect of an antioxidant such as L-carnitine against amiodarone pulmonary toxicity.

L-Carnitine (l-trimethylamino-β-hydroxybutyrate) is synthesized in vivo from methionine and lysine [12]. It is assumed that in normal circumstances, the biosynthesis of L-carnitine is sufficient to meet metabolic requirements, though in several disease situations (apart from primary carnitine deficiency)oral L-carnitine supplements may be necessary as therapy [13]. The primary function of L-carnitine is to act as a carrier for translocation of long-chain fatty acids from the cytosol into mitochondria for ß-oxidation, hence sustaining the supply of energy[14]. However, besides this well-known
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effect, there is growing evidence that L-carnitine also plays a role in other physiological processes in humans and animals. Indeed, L-carnitine act as very potent reactive oxygen species scavengers [15, 16] and are known to have immunomodulatory properties in mammalian as well as avian species [17].

L-carnitine has been reported as a glucocorticoid mimicker because it activates the intracellular glucocorticoid receptor-a and modulates the expression of glucocorticoid-dependent genes during inflammation [18-20]. Glucocorticoids have an suppressive effect on the synthesis of proinflammatory cytokines bymacrophages, and this effect was mimicked by L-carnitine[17]. Based on the aforementioned information, CAR has been selected in the present work as a possible protective agent. The rationale of the present study is to help in understanding the exact mechanism(s) of AMD-induced lung toxicity as well as the possible protective role of CAR against amiodarone lung toxicity. This may shed light on the usefulness of CAR as a safe natural product in such pathological situations.

MATERIALS AND METHODS

Chemicals

Amiodarone hydrochloride (an iodinated benzofuran derivative) was obtained from Sanofi Pharmaceuticals Company, France. Each 1 mg was dissolved in 10 mL of distilled water. L-carnitine was purchased from Sigma-Tau Pharmaceuticals, Pomezia, Italy. Thiobarbituric acid (TBA) was a product of Fluka (Buchs, Switzerland). All other chemicals were of the highest grade commercially available.

Animals:

Male Swiss albino rats weighing 150-200 g were used in all experiments. Animals were maintained under standard conditions of temperature & humidity with regular light/dark cycle and allowed free access to food (Purina Chow) and water. All animal experiments were conducted according to the regulations of the Committee on Bioethics for Animal Experiments of Riyadh colleges of dentistry and pharmacy.

Experimental Protocol:

The animals were divided at random into four groups of 10 animals each. The first group (control) received vehicles used for AMD (sterile water, i.p). The second group, was injected with CAR (200 mg/kg i.p) for 15 days [21]. The third group, was injected with amiodarone (AMD) (100 mg/kg i.p) for 10 days [22]. The fourth group, injected CAR ((200 mg/kg i.p) for 5 days before and 10 days concomitant with AMDas group 3. One day later, under light ether anesthesia, animals were sacrificed by cervical dislocation and the lungs were quickly isolated.

Calculation of Lung/Body Weight Coefficient:

Rats were weighed before being sacrificed (body weight). Their lungs were weighed after being removed. Lung/body coefficient was calculated as previously reported (lung/body coefficient = lung weight [g]/body weight [g] x 100%) [23].

Preparation of lung homogenates for biochemical measurements

The isolated left lungs were rinsed in chilled 1.15 % KCl (pH 7.4) and homogenization was carried out in ice-cold KCl (1.15%, pH 7.4) to yield a 10% (w/v) tissue homogenates.

Determination of lipid peroxides and glutathione content in lung homogenate:

Glutathione contents and lipid peroxidation (Malondialdehyde (MDA) production) in the lung tissues were determined according Ellman,1959 and Ohkawa et al., 1979 respectively[24,25].

Determination of enzyme activities of Glutathione reductase, catalase and Superoxide dismutase (SOD) in lung homogenate:

The enzyme activity ofGlutathione reductase (GSH-red), catalase and superoxide dismutase (SOD) were measured in the lung homogenates according Dolphin et al. (1989), Higgins et al., 1978 and McCord and Fridovich (1969) respectively .(27,28)

Determination of total nitrate/nitrite (NOx) concentrations in lung homogenate:

Total nitrate/nitrite (NO(x)) was measured as stable end product, nitrite, according to the method of Miranda et al. [29]. The assay is based on the reduction of nitrate by vanadium trichloride combined with detection by the acidic Griess reaction. The diazotization of sulfanilic acid with nitrite at acidic pH and subsequent coupling with N-(10 naphtyl)-ethylene diamine produced an intensely colored product that is measured spectro-photometrically at 540 nm. The levels of NOx were expressed as mol g−1 wet tissue.

Histopathology
Histopathological examination was performed on the animals of each group. Right lung samples were taken. The tissues were fixed for at least 48 hours in 10% formalin in phosphate buffer (pH 7). The samples were then embedded in paraffin wax, cut into 5 μm sections, and stained with hematoxylin-eosin. The slides were coded and were examined by a histopathologist who was unaware about the treated groups.

**Statistical Analysis**

Data are expressed as (means + SEM). Statistical comparison between different groups were done using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test to judge the difference between various groups. Significance was accepted at $P < 0.05$.

**RESULTS**

The Effect of Amiodarone on Rat Lung/Body Coefficient:

The lung weight was normalized to the body weight as a marker of oedema. Intraperitoneal administration of amiodarone (100 mg/kg/day) for 10 days resulted in a significant increase in lung/body coefficient compared to the control group. Pretreatment with CAR (200 mg/kg ip) for 5 days before and concomitantly with AMD injection before AMD injection prevented the significant increase in rat lung/body coefficient compared to AMD treated rats and control group (Fig 1).

**Figure 1**

Effects of CAR and AMD and their combination on the level of total lung/body coefficient

Lipid peroxides in lung homogenates, measured as malondialdehyde concentrations, of rats treated with amiodarone significantly decreased to 61% of the control value. Pretreatment with CAR (100 mg/kg ip) for 5 days before and concomitantly with AMD injection prevented the significant decrease in lung malondialdehyde concentrations (Fig 2). Amiodarone injection produced a significant reduction of reduced glutathione in lung homogenates (0.43+ 0.04 umol/g tissue) as compared with the control value (1.24+0.16 umol/g tissue). Pretreatment with CAR for 5 days before and concomitantly with AMD injection prevented the significant depletion of lung GSH content compared to AMD treated rats [Fig 3]. On the other hand administration of CAR alone did not produce any significant change in the reduced glutathione content and lipid peroxides in lung tissues.

**Figure 2**

Effect of CAR, AMD and their combination on the levels of thiobarbituric acid reactive substance (TBARS) in rat lung tissues

**Figure 3**

Effect of CAR, AMD and their combination on the levels of reduced glutathione in rat lung tissues

Effect of CAR and AMD on lipid peroxides and reduced glutathione content in lung homogenate:
Effect of CAR and AMD on the total nitrate/nitrite (NOx) concentrations in lung tissues

Fig. 4 shows the effects of CAR, AMD and their combination on nitrosative stress biomarkers NOx (C) in lung tissues. AMD resulted in a significant increase in NOx, in lung tissues to reach 114% of the control value. Administration of CAR alone showed non-significant change. Combined treatment with CAR and AMD resulted in a significant decrease in NOx in lung tissues as compared to AMD group.

**Figure 4**
Effect of CAR, AMD and their combination on the level of total nitrate/nitrite (NOx) levels in rat lung tissues

Effect of CAR and AMD on antioxidant enzymes:

AMD treatment resulted in a significant increase of glutathione reductase enzyme activities to reach 127% of the control value. Combined treatment with CAR and AMD resulted in a significant decrease of this enzyme as compared to AMD group (Fig 5). AMD treatment resulted in a significant decrease of catalase and superoxide dismutase enzymes activities to reach 70% and 52% of the control value respectively. Combined treatment with CAR and AMD resulted in a complete reversal of AMD-induced changes to the control values. On the other hand administration of CAR alone did not produce any significant changes in the antioxidant enzymes activities. (Figs. 6, 7).

**Figure 5**
Effects of CAR and AMD and their combination on the level of Glutathione reductase

**Figure 6**
Effect of CAR, AMD and their combination on the level of catalase activity in rat lung tissues

**Figure 7**
Effects of CAR and AMD and their combination on the level of superoxide dismutase enzyme

**Lung Pathology**

Histopathological examination of the lungs of control, and
CAR groups showed normal histological structure of the lung. The interalveolar septa were seen to be thin and the alveoli and alveolar sacs appeared clear and patent (Fig. H1). However, animals treated with AMD showed loss of normal lung architecture including loss of continuity of alveolar epithelium, extensive thickening and distortion of the interalveolar septa with dilatation and congestion of the pulmonary blood vessels and perivascular and peribronchial cellular infiltration (Figs. H2, H3). Interestingly, lung specimens from rats treated with CAR and AMD revealed significant preservation of normal architecture, nearly similar to the control group (Fig. H4).

**Figure H1**
A Histopathology of rat lung of the control group showing normal lung architecture with thin inter-alveolar septa, normal clear alveoli and alveolar sacs. Hx. & E.; X 200

**Figure H21**
A Histopathology of rat lung of the AMD treated group showing loss of continuity of alveolar epithelium, congestion and dilatation of the pulmonary blood vessels with perivascular and interstitial mononuclear cellular infiltration in the form of nodules. Hx. & E.; x 200

**Figure H3**
A Histopathology of rat lung of the AMD treated group showing interstitial and peribronchiolar mononuclear cellular infiltration, with focal areas of overexpansion with destroyed interalveolar septa, congested blood vessel. Hx. & E.; x 200
Figure H4
A Histopathology of rat lung rat from CAR and AMD treated group showing preserved normal architecture with decrease in the interalveolar thickness to be more or less as control group but with little few thickened infiltrated parts. Hx. & E.; x 400

DISCUSSION
Amiodarone is a highly effective antidysrhythmic agent. However, it is associated with many side effects involving many different organ systems (30). The most serious side effect of amiodarone is pulmonary toxicity. Lung/body coefficient as marker of pulmonary oedema [31] and as conventional toxicological method was successfully used to determine the level of lung injury. The results of the present study revealed that daily administration of amiodarone (100 mg/kg/day i.p) for ten days resulted in a significant increase in lung/body coefficient. Similar findings were reported [32,33].

Amiodarone (100 mg/kg/day i.p) for ten days produce significant decrease in MDA level compared to the control group. Similar results were reported [34-37]. Card et al., found that formation of thiobarbituric acid reactive substances (TBARS) was not altered by amiodarone in incubations lasting to 1 hour [38]. This report indicated that lipid peroxidation, as indicated by levels of TBARS, does not play a role in amiodarone-induced alteration in mitochondrial respiration and membrane potential. Moreover, in vitro exposure of isolated hamster lung mitochondria to amiodarone did not induce lipid peroxidation [39]. Previous studies on the effect of AMD on lipid peroxidation in rat liver mitochondria, measured by TBRAS, reported that it provoked inhibition of peroxidation [40]. The same authors suggested a protective effect of AMD against lipid peroxidation in mitochondrial membranes challenged by iron dependent system. This inhibition could be situated at the level of initiation or propagation of peroxidation.

Nitric oxide (NO) has been associated with oxidant related tissue injury by formation of highly reactive nitrogen intermediates via interactions with ROS. High concentration of NO has direct toxic effects. NO also reacts with superoxide and generates a highly active metabolite, peroxynitrite, which is presumed to be largely responsible for most of the adverse effects of excessive generation of NO [41].

In the present study, changes of NO (nitrate/nitrite) were monitored. The results presented clearly demonstrate that AMD administration of resulted in a significant increase in nitrate and nitrite concentration in the rat lung homogenate. Kishida et al. (2006) provided the first evidence that AMD and its metabolites, enhance eNOS (endothelial nitric oxide synthase)–mediated NO production in human endothelial cells[42]. Nitric oxide increases cell proliferation in cultured human fibroblasts[43]. (Gansauge et al., 1997)

Reduced glutathione (GSH) plays an important role in a variety of detoxification processes. In the present study AMD decreased significantly the GSH level in rat lung homogenate, suggesting a role of oxidative stress in AMD induced lung toxicity. The major enzymatic antioxidants of the lungs are superoxide dismutases and catalase. These antioxidants are the first lines of defense against the oxidants and usually act at a gross level [22].

In the current study, administration of AMD resulted in a significant decrease in the level of catalase and SOD together with a significant increase in glutathione reductase activity in rat lung homogenate. A direct role for oxidative stress in AMD induced lung toxicity has been proposed [44]. The elevation of lung glutathione reductase activity observed in the present study following daily administration of AMD for ten days is in agreement with the previous finding of Leeder et al. (1996) who reported a similar elevation in lung glutathione reductase level and stated that such elevation most likely represents inflammation of lung and influx of glutathione reductase-containing inflammatory cells rather than an adaptive induction due to oxidative stress [44]. Histopathological finding of the lung following administration of amiodarone showed vascular congestion and interstitial capillary dilatation with moderate lymphocytic infiltrate.
L-carnitine is a co-factor essential for the beta oxidation of long chain fatty acids by providing the translocation of fatty acids into the mitochondrial matrix and also a buffer for the potentially toxic acyl-Co A [45]. L-carnitine has been shown to ameliorate several different models of toxicity cases like doxorubicin-induced cardiotoxicity [46], methamphetamine neurotoxicity [47], simvastatin cytotoxicity [48]. Another way of protection by L-carnitine might be related to increasing antioxidant capacity in cellular mitochondria [49]. Our results demonstrated that CAR significantly attenuated the development of AMD induced lung toxicity. Further studies are needed to elucidate the exact mechanisms of this protection and to study the possible protective effect of CAR against other pneumotoxicants.

References
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