European Journal of Pharmacology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

L-carnitine preserves cardiac function by activating p38 MAPK/Nrf2 signalling in hearts exposed to irradiation

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ARTICLE INFO

Keywords:
L-carnitine
Radiation-induced heart damage
Cardiac function
Apoptosis
Reactive oxygen species

ABSTRACT

Radiation-induced heart damage (RIHD) is now considered to be one of the causes of mortality in cancer patients undergoing radiotherapy. Cardiac function impairments are clinical manifestations of RIHD. L-carnitine shows protective effects against irradiation and heart disease. This study was aimed to investigate the cardioprotective effects and potential molecular mechanisms of L-carnitine against RIHD. Mouse hearts were exposed to γ-radiation to induce RIHD. L-carnitine at doses of 100 mg/Kg and 200 mg/Kg was used to treat animals intraperitoneally. Additionally, a specific inhibitor of p38 MAPK was used to treat animals by intraperitoneal injections. Cardiac systolic/diastolic functions were determined using invasive hemodynamic methods; myocyte apoptosis was assessed using the TUNEL assay; intracellular reactive oxygen species production was measured using DHE staining; and western blotting was used to evaluate the phosphorylation of p38MAPK, phosphorylation of Nrf2, and expression levels of HO1, NQO1, caspase3 and bax. L-carnitine treatments inhibited irradiation induced cardiac function impairments. Radiation exposure induced myocyte apoptosis and reactive oxygen species production, which were attenuated by L-carnitine treatments. However, administration of a p38 MAPK inhibitor (SB203580) dramatically impaired L-carnitine's effect on attenuating apoptosis, reactive oxygen species accumulation and cardiac functions in irradiated hearts. Our study showed that L-carnitine administration activated p38MAPK/Nrf2 signalling, initiating the expression of HO1 and NQO1, which have anti-apoptotic and anti-oxidative effects, respectively. In conclusion, L-carnitine attenuates cardiac function loss by inhibiting reactive oxygen species production and apoptosis in hearts exposed to radiation. The cardioprotective effects of L-carnitine were mediated by p38MAPK/Nrf2 signalling.

1. Introduction

Many patients with breast, pulmonary and thoracic cancer receive radiation therapy. Radiation-induced heart disease (RIHD) is a complication of radiation therapy in these patients (Tapio, 2016). Due to cardiac tissue toxicity, it has been reported that RIHD contributes to the mortality of malignant cancers (Taunk et al., 2015). RIHD is defined as functional and structural heart damage caused by high-dose radiation exposure. Common pathological changes that characterize RIHD include myocardium fibrosis, coronary artery injury, pericardial adhesions, microvascular damage and the loss of contractile units (Boerma, 2012). Therefore, a widely used, clinically safe treatment that exerts cardioprotective effects would provide an effective strategy for preventing and treating RIDH.

L-carnitine, a small water- soluble natural amino acid, plays a role in transporting long-chain fatty acid molecules from the cytoplasm to the mitochondrial matrix, where β- oxidation of fatty acids occurs (Tousson et al., 2016). Also known as L- trimethyl 1–3-hydroxy ammoniobetanoate, L-carnitine and its derivatives are believed to be anti-inflammatory and antioxidant agents (Mishra et al., 2016). Recent investigations have noted that L-carnitine is an important anti-apoptotic factor (Ishii et al., 2000). Supplementation with L-carnitine was found to enhance carbohydrate metabolism and inhibit the accumulation of toxic metabolites under ischemic conditions (Lango et al., 2001). L-carnitine deficiency is associated with heart failure and myocardial infarction (Lee et al., 2011; Pauly and Pepine, 2003). L-carnitine is now recognized as a cardioprotective medication (Oyanagi et al., 2011). Moreover, *in vivo* and *in vitro* studies have shown that L-carnitine preserves organ function and reduces radiation- induced tissue damage by inhibiting cell apoptosis (Akpolat et al., 2013; Kanter et al., 2010). Thus, it is reasonable for us to speculate that L-carnitine may preserve the cardiac pumping function by reducing contractile

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http://dx.doi.org/10.1016/j.ejphar.2017.04.003
Received 10 September 2016; Received in revised form 29 March 2017; Accepted 5 April 2017

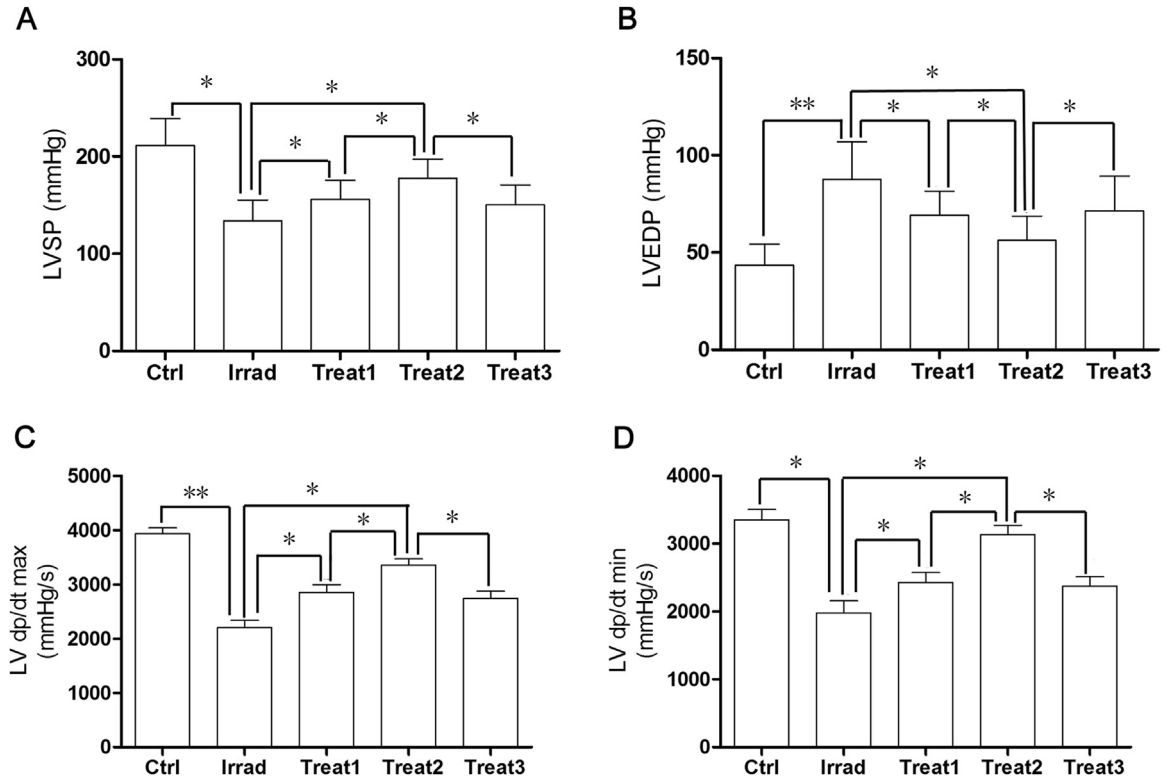

Fig. 1. Cardiac functions were determined by invasive, cardiac hemodynamic assessments. The measured LVSP, LVEDP, LV dp/dt max and LV dp/dt min are shown as columns in A, B, C and D, respectively. Columns (from left to right) represent the measured values in mice from the Ctrl, Irrad, Treat1, Treat2, and Treat3 groups, respectively. (*P < 0.05, **P < 0.01).

unit loss in hearts exposed to radiation.

Oxidative stress is considered to be the most direct and critical factor among the mechanisms involved in irradiation-induced cell apoptosis. Injured mitochondria generate excess reactive oxygen species (ROS) through maladjusted mitochondrial oxidation (Wang et al., 2015). Intracellular reactive oxygen species activates p38 MAPK (mitogen- activated protein kinase), an oxidative sensor that belongs to the MAPK family (Werner et al., 2014). Activated p38 MAPK regulates its down-stream transcriptional factors, which further modulate apoptotic events. By promoting the phosphorylation of NF-E2- related factor2 (Nrf2), activated p38 MAPK could facilitate the disassociation of Nrf2 from Keap1 to initiate the transcription of several anti-apoptotic and anti- oxidant genes, such as heme oxygenase-1 (HO1) and quinine oxidoreductase-1 (NQO1) (Kim et al., 2015; Zhu et al., 2016).

In this study, cardiac function and contractile loss were investigated in mice that were exposed to local thoracic radiation. The curative effects of L-carnitine supplementation were observed. The mechanisms of L-carnitine's cardioprotective effects, which involve reactive oxygen species /p38 MAPK signalling, were also investigated. These results provide us with knowledge about radiation-induced cardiac damage as well as a theoretical basis for the clinical applications of L-carnitine in patients undergoing radiation therapy.

2. Material and methods

2.1. Animals and irradiation exposure protocol

Male C57Bl/6j mice (10-week old) provided by the Animal Experimental Centre of Xi'an Jiaotong University were used in this study. Animals were raised in isolated cages in temperature- controlled rooms and were provided continuous 12 h artificial dark-light cycles. Animals received standard mouse chow and had free access to tap water. The irradiation protocol was implemented in accordance with descriptions in previous reports but with some modifications (Gabriels et al., 2012). Mice were anesthetized with chloral hydrate (10%, v/v), and radiation was delivered using a cobalt-60 (^{60}Co) teletherapy unit (Cirus). The mouse hearts were exposed to a γ-radiation field of 5 mm×5 mm. The total dose of γ-radiation was 3 Gy/day for each animal for 5 consecutive days.

2.2. Experimental design

Fifty experimental mice were divided evenly and randomly into 5 groups. The control group (Ctrl) received sham radiation and an intraperitoneal injections of 0.2 ml of saline before irradiation; the irradiation group (Irrad) received γ-irradiation and intraperitoneal injections of 0.2 ml of saline before irradiation; treatment group 1 (Treat 1) received γ-irradiation and intraperitoneal injections of L-carnitine (Sigma-Aldrich) of 100 mg/Kg before exposure; treatment group 2 (Treat 2) received γ-irradiation and intraperitoneal injections of L-carnitine (Sigma-Aldrich) of 200 mg/Kg before exposure; treatment group 3 (Treat 3) received γ-irradiation, intraperitoneal injections of L-carnitine (Sigma-Aldrich) of 200 mg/Kg in addition to 15 mg/Kg p38 MAPK inhibitor SB203580 (LC Laboratories) before exposure.

2.3. Invasive cardiac hemodynamic detection

The cardiac systolic/diastolic functions were evaluated with an invasive hemodynamic method. The protocol was in accordance with previous studies. Briefly, mice were anesthetized by intraperitoneal injections of chloral hydrate (10%, v/v). A Mikro Tip catheter (Millar Instruments) was intubated into the left ventricle through the right carotid artery. The catheter was then connected to a pressure transducer attached to the Powerlab Biological Analysis System (AD Instruments). The left ventricular systolic pressure (LVSP), left ventricular end- diastolic pressure (LVEDP), maximum rate of left ventricular pressure increase (LVdp/dt max) and maximum rate of left ventricular pressure decrease (LVdp/dt min) were measured.

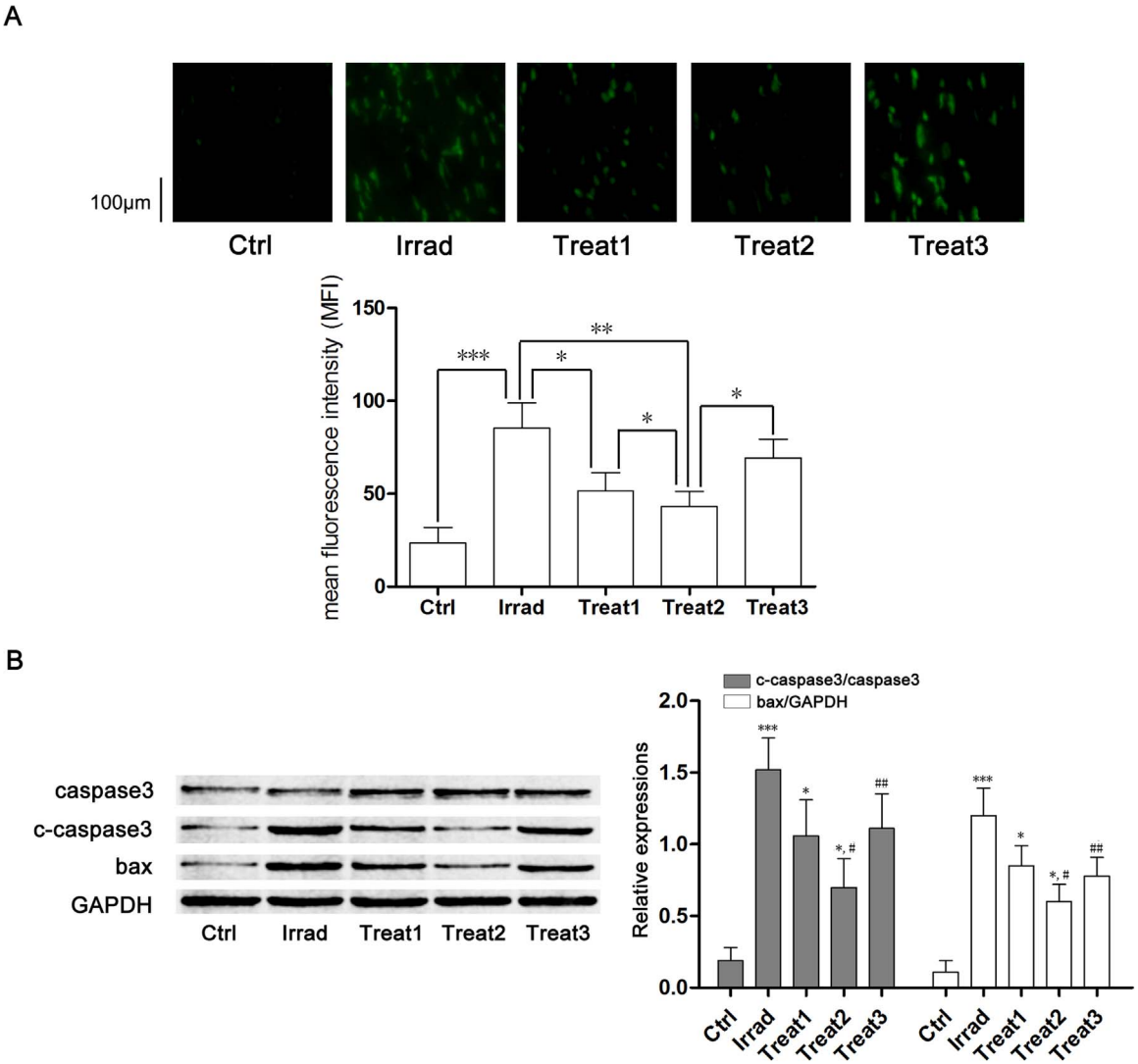

Fig. 2. (A) The top column of this figure shows the captured images from the TUNEL assay of cardiac tissue from Ctrl, Irrad, Treat1, Treat2 and Treat3, respectively. The bottom columns show the mean fluorescence intensity of TUNEL assay (*P < 0.05, **P < 0.01, *** P < 0.001). (B) The left side of this figure shows the caspase3, cleaved caspase3, bax and GAPDH immunoblots of cardiac samples from Ctrl, Irrad, Treat1, Treat2 and Treat3, respectively. The grey columns on the right side of this figure show the relative expression levels of cleaved caspase3 (normalized to caspase3) in each group. The white columns on the right side of this figure show the relative expression levels of bax (normalized to GAPDH) in each group. (***) differences were statistically significantly when compared with Ctrl, P < 0.001; (*) differences were statistically significantly when compared with Irrad, P < 0.05; (#) differences were statistically significantly when compared with Treat1, P < 0.05; (##) differences were statistically significantly when compared with Treat2, P < 0.05.

2.4. Myocardial apoptosis

Cell apoptosis was detected using the terminal dUTP transferase nick end labelling (TUNEL) assay. The process was carried out according to the protocol provided by the manufacturer. Briefly, left ventricular cardiac tissue was harvested and embedded in paraffin. Subsequently, the tissues were sectioned into slides, deparaffinized and digested using proteinase K (Sigma-Aldrich). The samples were washed in PBS, and the TUNEL assay kit (Roche) was used to tag apoptotic myocytes. Tagged cells were observed with an inverted fluorescence microscope. A randomization method was used to reduce bias when selecting the regions of heart for imaging.

2.5. In situ *detection of reactive oxygen species*

In situ reactive oxygen species detection was performed using DHE staining. After the harvested left ventricular cardiac tissue was embedded in OCT (Sakura), 10-μm thick cryostat sections were made at −20 ℃. The slides were incubated in DHE (Beyotime) at a final concentration of 10 μmol/l at 37 ℃ for 45 min in a dark chamber. Fluorescent images were captured using an inverted fluorescence microscope. A randomization method was used to reduce bias when selecting the regions of the heart for imaging.

2.6. Western blotting

Harvested cardiac tissue was minced and lysed using the RIPA lysis buffer system (Santa Cruz) supplemented with PMSF (Invitrogen). After centrifugation at 12,500*g* and 4 ℃ for 15 min, the supernatants were separated. Total protein was extracted with a Protein Extraction kit (Beyotime) according to the manufacturer's instructions. A BCA assay kit (Pierce) was used to detect the concentrations of the total protein samples. SDS-PAGE was used to separate the proteins, which were then transferred to polyvinylidene fluoride (PVDF) membranes. After incubation with blocking buffer, the membranes were incubated with specific primary antibodies against p38 (CST, #14451, 1:2000), phospho-p38 (CST, #4551, 1:2000), cleaved caspase3 (Abcam, #ab2302, 1:4000), caspase3 (Abcam, #ab2141, 1:4000), bax (Abcam, #ab32503, 1:2000), Nrf2 (Abcam, #ab76026, 1:2000), phospho-Nrf2 (Abcam, #ab180844, 1:2000), HO1 (Invitrogen, #ab13248, 1:2000),

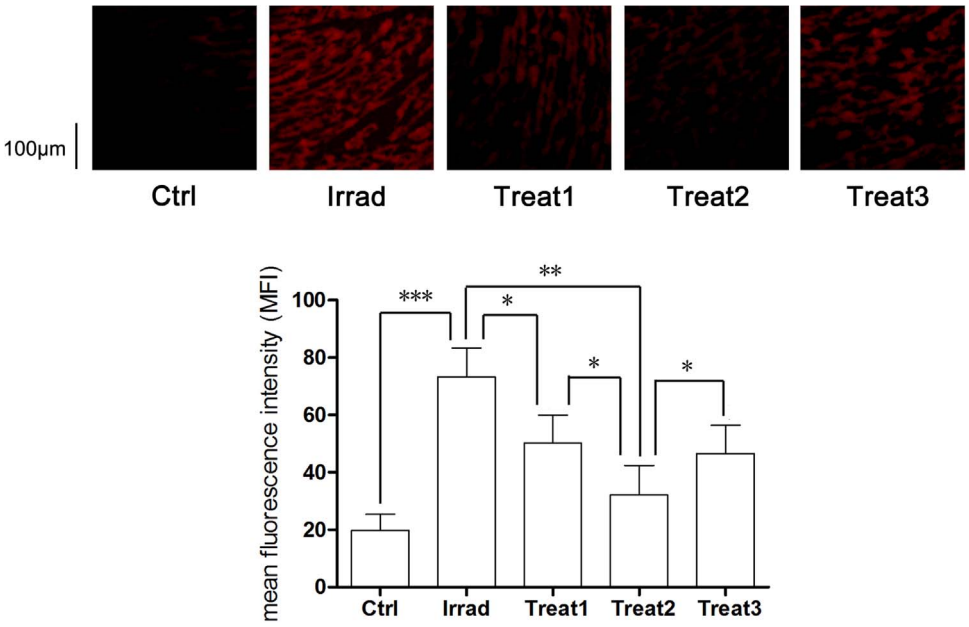

Fig. 3. The top panel of this figure shows the captured images following DHE staining of cardiac tissue from Ctrl, Irrad, Treat1, Treat2 and Treat3, respectively. The bottom columns show the mean fluorescence intensity of DHE staining (*P < 0.05, **P < 0.01, *** P < 0.001).

NQO1 (Abcam, ab28947, 1:2000) and GAPDH (Abcam, #ab8245, 1:5000). The membranes were washed with Tris buffered saline (TBS) and incubated with the corresponding HRP- conjugated secondary antibodies. The immunoblots were visualized on X-ray films using the Super Signal West Pico chemiluminescence reagent (Thermo Scientific).

2.7. Statistics

Data collected in this study were expressed as the mean ± SD. The data were analysed using the SPSS software (v.17.0, SPSS) using Student's *t*-tests and one-way analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

3. Results

3.1. L-carnitine preserved cardiac function after radiation exposure

Cardiac functions were assessed using invasive hemodynamic measurements in this study. After radiation exposure, cardiac functions were impaired, as evidenced by elevated LVEDP, reduced LVSP, LVdP/dt max and LVdp/dt min. However, cardiac functions were preserved in mice that received L-carnitine injections. L-carnitine at doses of 200 mg/Kg was superior at preserving cardiac functions than L-carnitine at doses of 100 mg/Kg. The results are shown in Fig. 1.

3.2. L-carnitine decreased irradiation- induced myocardium apoptosis in a concentration- dependent manner

The captured images from the TUNEL assay are shown in Fig. 2. Cell apoptosis dramatically increased in hearts exposed to radiation. However, L-carnitine treatments significantly suppressed apoptosis of myocytes. L-carnitine showed a concentration- dependent inhibitory effect on apoptosis. Moreover, the expression levels of bax and cleaved caspase3 increased significantly after radiation exposure, which was decreased by L-carnitine administration.

3.3. L-carnitine reduced intracellular reactive oxygen species generation in cardiac tissue after irradiation in a concentration-dependent manner

Captured images from DHE fluorescence staining are shown in Fig. 3. DHE was used as a specific indicator of reactive oxygen species. After radiation exposure, excess reactive oxygen species were generated in cardiac tissue. However, L-carnitine showed antioxidant activity by lowering the intracellular reactive oxygen species generation in hearts exposed to radiation. This effect was found to be concentration-dependent.

3.4. L- carnitine promoted p38 MAPK signalling activation in hearts exposed to radiation

The results are shown in Fig. 4. L-carnitine administration significantly increased the p38 MAPK signalling activity in irradiated hearts. The p38 MAPK phosphorylation, Nrf2 phosphorylation, HO1 expression and NQO1 expression levels were elevated after the administration of L-carnitine to irradiated hearts.

3.5. p38 MAPK inhibitor administration impaired L-carnitine's antioxidant and cardioprotective effects

The effects of SB203580, a p38 MAPK specific inhibitor, on cardiac function, apoptosis, reactive oxygen species generation and p38 MAPK signalling activation are shown in Figs. 1–4. SB203580 administration dramatically impaired cardiac function preservation as well as the antioxidant and anti-apoptotic activities of L-carnitine in radiation exposed hearts. Moreover, activation of p38 MAPK signalling induced by L-carnitine was inhibited due to SB203580 administration.

4. Discussion

In the present study, we investigated cardiac damage induced by a sub-lethal dosage of irradiation. Additionally, L-carnitine, a cardioprotective agent, was used to treat the animals. The molecular mechanisms of the cardioprotective activity of L-carnitine were studied. First, we used a modified irradiation exposure protocol to treat animals. The dosage of local radiation was 3 Gy for 5 consecutive days. The results

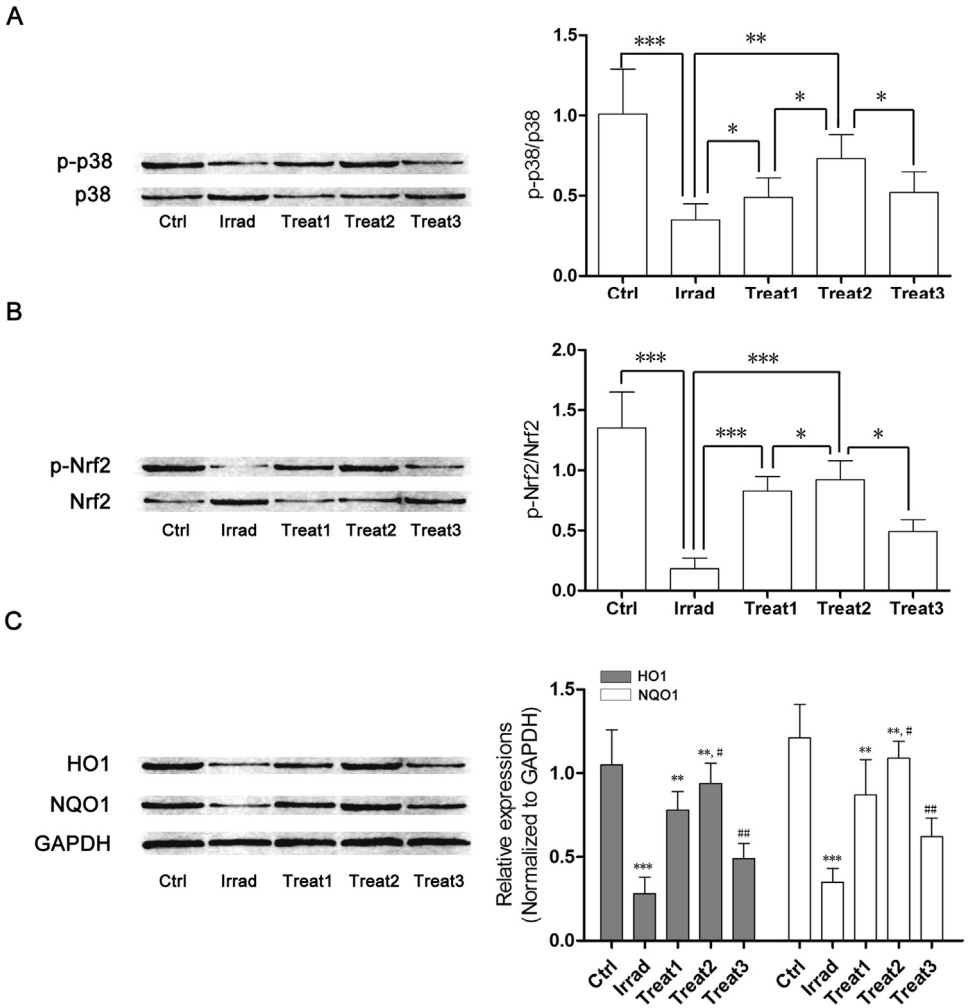

Fig. 4. (A) The left column shows p-p38 and p38 immunoblots of cardiac tissue from Ctrl, Irrad, Treat1, Treat2 and Treat3, respectively. The columns on the right show the relative expression levels of p-p38 (normalized to p38) in each group (*P < 0.05, **P < 0.01, *** P < 0.001). (B) The left column shows the p-Nrf2 and Nrf2 immunoblots of cardiac tissue from Ctrl, Irrad, Treat1, Treat2 and Treat3, respectively. The columns on the right show the relative expression levels of p-Nrf2 (normalized to Nrf2) in each group (*P < 0.05, *** P < 0.001). (C) The left column shows the HO1, NQO1 and GAPDH immunoblots of cardiac tissue from Ctrl, Irrad, Treat1, Treat2 and Treat3, respectively. The columns on the right show the relative expression levels of HO1 and NQO1 (normalized to GAPDH) in each group. (***) differences were statistically significantly when compared with Ctrl, P < 0.001; (**) differences were statistically significantly when compared with Irrad, P < 0.01; (#) differences were statistically significantly when compared with Treat1, P < 0.05; (##) differences were statistically significantly when compared with Treat2, P < 0.01.

showed that the cardiac systolic/diastolic functions were significantly impaired due to irradiation. Second, elevated intracellular reactive oxygen species production and myocyte apoptosis were identified in irradiated hearts. Third, L-carnitine treatments dramatically preserved cardiac function by reducing reactive oxygen species production and myocyte apoptosis. Moreover, activation of the p38 MAPK signalling pathway was proposed as the molecular mechanism underlying L-carnitine's cardioprotective effects against irradiation- induced cardiac damage.

RIHD is defined as the functional and structural cardiac damage after a heart is exposed to high dose of radiation. In recent decades, an increasing number patients with malignant tumours have received radiotherapy. RIHD has a relatively high incidence in patients with thoracic and mediastinal cancers who receive radiotherapy. Deterioration of cardiac functions is identified as one of the common pathological manifestations of RIHD (Tapio, 2016). In this study, using invasive cardiac hemodynamic assessments, we found that after irradiation, LVEDP increased, whereas LVSP decreased significantly, indicating that both systolic and diastolic functions were impaired. Moreover, the decreased values of LVdP/dt max and LVdP/dt min indicated that cardiac compliance was impaired after irradiation. It has been shown that irradiation injures exposed tissue through oxidative stress (Jeena et al., 2016). Radiation exposure could lead to mitochondrial dysfunction, which further generates excessive reactive oxygen species through deregulated oxidation (Liu et al., 2016). It was reported that irradiation undermined the anti-oxidant defence mechanism in exposed hearts, leading to the accumulation of free radicals (Wang et al., 2016). In the present study, we found that excess intracellular reactive oxygen species were generated in hearts exposed to radiation. As evidenced by the TUNEL assay and caspase cascade activation data, apoptosis of myocytes was dramatically increased after irradiation. The significant loss of contractile units could explain the impairment of cardiac function.

L-carnitine shows a wide spectrum of biological activities, including anti- inflammation, immunoregulation, neuroprotection, gastroprotection and cardioprotection (Khan and Alhomida, 2011). Some previous studies have shown that L-carnitine exhibited protective effects in irradiation-induced tissue damage in bone marrow, crystalline tissues, oral mucosa and the testicles (Ahmed et al., 2014). L-carnitine has been shown to act as a free radical scavenger and anti-apoptotic mediator (Hua et al., 2015). A previous study demonstrated that L-carnitine could reduce 2.45-GHz radiation-induced oxidative stress in hearts (Turker et al., 2011). In this study, animals exposed to γ-irradiation were treated with L-carnitine. Our results showed that L-carnitine

administration significantly improved cardiac systolic/diastolic functions as well as cardiac compliance. Furthermore, apoptosis and intracellular reactive oxygen species accumulation were found to be dramatically decreased in irradiated hearts harvested from animals that received L-carnitine treatments. These results indicated that by reducing irradiation- induced cardiac damage, L-carnitine was a potential cardioprotective agent in patients undergoing radiotherapy.

Although several mechanisms have been previously studied, the exact mechanisms of L-carnitine's cardioprotective role are not completely understood. Therefore, we further investigated the underlying molecular mechanisms. A p38 MAPK specific inhibitor, SB203580, was used to treat animals simultaneously along with L-carnitine. The animals were then exposed to γ-radiation. The results indicated that L-carnitine's cardioprotective effects were significantly impaired. SB203580 and L-carnitine co-treatment decreased cardiac functions, increased myocardial apoptosis and elevated intracellular reactive oxygen species accumulation compared to L-carnitine treatments. These results indicate that p38 MAPK was the molecular target of L-carnitine. We found that in irradiated hearts, L-carnitine treatments significantly increased p38 MAPK phosphorylation. As a result, the phosphorylation levels of Nrf2, a downstream mediator of p38 MAPK, also increased. Activated Nrf2 disassociates from Keap1 and then translocates to the nucleus, where it initiates transcription of several genes, such as HO1 and NQO1 (Ryu and Chung, 2016; Shanmugam et al., 2016). The anti-oxidant and anti-apoptotic roles of HO1 and NQO1 have been clearly established (Huang et al., 2016; Siew et al., 2012). We found that L-carnitine treatments dramatically increased the phosphorylation levels of Nrf2, and correspondingly, the expression levels of HO1 and NQO1 were also increased. We found that co-treatment of L-carnitine with SB203580 decreased the activation of the p38 MAPK- Nrf2- HO1/NQO1 signalling pathway. These results established that L-carnitine exerted cardioprotective effects through p38 MAPK.

In summary, our present observations indicated the potent protective effects of L-carnitine on hearts exposed to γ-radiation. Further investigation of the mechanisms revealed that p38 MAPK was the molecular target of L-carnitine. By activating p38 MAPK- Nrf2 signalling, L-carnitine exerted cardioprotective effects by suppressing myocyte apoptosis and intracellular reactive oxygen species accumulation in radiation exposed hearts. The present study provided additional evidence supporting the beneficial effects of L-carnitine in preventing and treating RIHD, suggesting the potential clinical applications of L-carnitine in patients with thoracic and mediastinal cancers who receive radiotherapy.

Ethics approval

All animal experimental procedures were carried out according to the recommended guidelines for the care and use of laboratory animals issued by the Chinese Council on Animal Research. The protocol on animal experiments was approved by the Ethics Committee on Human and Animal Use of Xi'an Jiaotong University.

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