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SHORT COMMUNICATION

Protective effects of mito-TEMPO against doxorubicin cardiotoxicity in mice

Viviane Costa Junqueira Rocha[1] · Luciana Souza de Aragão França[1] · Cintia Figueiredo de Araújo[1] · Ayling Martins Ng[1] · Candace Machado de Andrade[1] · André Cronemberger Andrade[2] · Emanuelle de Souza Santos[1] · Mariana da Cruz Borges-Silva[1] · Simone Garcia Macambira[1] · Alberto Augusto Noronha-Dutra[1,3] · Lain Carlos Pontes-de-Carvalho[1]

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Abstract
Purpose Doxorubicin (DOX) is a chemotherapeutic that is widely used for the treatment of many human tumors. However, the development of cardiotoxicity has limited its use. The aim of the present study was to evaluate the possible efficacy of mito-TEMPO (mito-T) as a protective agent against DOX-induced cardiotoxicity in mice.
Methods C57BL/6 mice were treated twice with mito-T at low (5 mg/kg body weight) or high (20 mg/kg body weight) dose and once with DOX (24 mg/kg body weight) or saline (0.1 mL/20 g body weight) by means of intraperitoneal injections. The levels of malondialdehyde (MLDA), a marker of lipid peroxidation, and serum levels of creatine kinase were evaluated 48 h after the injection of DOX.
Results DOX induced lipid peroxidation in heart mitochondria ($p < 0.001$), and DOX-treated mice receiving mito-T at low dose had levels of MLDA significantly lower than the mice that received only DOX ($p < 0.01$). Furthermore, administration of mito-T alone did not cause any significant changes from control values. Additionally, DOX-treated mice treated with mito-T at high dose showed decrease in serum levels of total CK compared to mice treated with DOX alone ($p < 0.05$).
Conclusion Our results indicate that mito-T protects mice against DOX-induced cardiotoxicity.

Keywords Doxorubicin · Cardiotoxicity · Mitochondria · Mito-TEMPO

Introduction

Doxorubicin (DOX) is an anthracycline chemotherapeutic that is widely used for the treatment of many human tumors since the late 1960s [1]. Its discovery represented one of the great advancements in the fight against cancer; however, the development of adverse drug reactions, in particular cardiotoxicity, has limited its use [2].

Although DOX-induced cardiac toxicity appears to be multifactorial, the most thoroughly investigated hypothesis has been the formation of reactive oxygen species (ROS) and there is evidence pointing to cardiac mitochondria as primary targets of the toxicity of DOX [3]. The quinone moiety of DOX may form semiquinone radicals by one-electron reduction. This semiquinone, in the presence of molecular oxygen, results in the formation of superoxide anion and other ROS. Furthermore, it has been reported that DOX shows a high affinity for cardiolipin, a phospholipid in the inner mitochondrial membrane, which results in their accumulation inside cardiac cells and oxidative damage [4].

The possibility that cardiac dysfunction may lead to congestive heart failure stimulates the development of strategies to prevent or reduce DOX cardiotoxicity in the clinic, and the use of antioxidants could be an important strategy [5].

The mito-TEMPO (mito-T) is a nitroxide conjugated with a triphenylphosphonium (TPP) moiety that is

✉ Lain Carlos Pontes-de-Carvalho
lain.carvalho@gmail.com

[1] Gonçallo Moniz Research Center, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, Salvador, BA 40296-710, Brazil

[2] Laboratório de Imunologia Celular e Bioquímica de Fungos e Protozoários, Universidade Federal de São Paulo, São Paulo, SP, Brazil

[3] University College London, Gower Street, London, UK

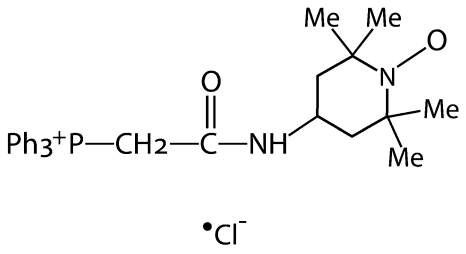

Fig. 1 Chemical structure of mito-TEMPO ($C_{29}H_{35}N_2O_2P{\cdot}Cl$)

mitochondria-targeted and have been used as antioxidant. Since nitroxides are known to be superoxide dismutase (SOD)-mimetics, mito-T may act as a mitochondrial superoxide scavenger and to protect mitochondria from the DOX-induced oxidative damage [6]. Recent studies documented that mito-T decreased mitochondrial superoxide levels and increased survival rate from septic mice [7], attenuated TNF-α-induced mitochondrial ROS and apoptosis in H9c2 cells [8] and blocked mitochondrial ROS generation and reduced DOX-induced platelet apoptosis [9].

Thus, as DOX produces significant amounts of ROS and has affinity for mitochondria, inducing mitochondrial and cardiac oxidative damage, the aim of the present study was to evaluate the possible efficacy of mito-T as a protective agent against DOX-induced cardiotoxicity in mice.

Materials and methods

Animals and ethical approval

Six- to ten-week-old female C57BL/6 mice were obtained and maintained at the animal facilities at the Gonçalo Moniz Research Center-FIOCRUZ (Salvador, Bahia, Brazil). The animals received balanced feed and water ad libitum.

The current work was carried out in accordance with the Brazilian Federal Law on Animal Experimentation (Law 11794) (http://www.planalto.gov.br/ccivil_03/_ato2007-2010/2008/lei/l11794.htm). The protocol was approved by the Ethics Committee for the Use of Animals in Research (CPqGM-FIOCRUZ, CEUA, license number 019/2012).

Design of the work

Seven to eight mice from each group were treated twice with mito-T (Fig. 1; Sigma-Aldrich Chemical Co, St. Louis, MO, USA) at low (5 mg/kg body weight) or high (20 mg/kg body weight) dose and once with DOX (Glenmark Farmacêutica Ltda, São Paulo, SP, Brazil; 24 mg/kg body weight) or saline (Farmace, Barbalha, CE, Brazil; 0.1 mL/20 g body weight) by means of intraperitoneal injections, as described below. The mice were euthanized 48 h after DOX by carbon dioxide inhalation.

Low dose of mito-T:

Group 1—Saline and saline;
Group 2—mito-T (5 mg/kg) twice, 48 and 24 h before saline;
Group 3—mito-T (5 mg/kg) twice, 48 and 24 h before DOX (24 mg/kg);
Group 4—DOX (24 mg/kg).

High dose of mito-T:

Group 1—Saline and saline;
Group 2—mito-T (20 mg/kg) twice, 24 and 1 h before saline;
Group 3—mito-T (20 mg/kg) twice, 24 and 1 h before DOX (24 mg/kg);
Group 4—DOX (24 mg/kg).

Determination of lipid peroxidation in heart mitochondria

Hearts of animals treated with low dose of mito-T were collected and mitochondria obtained as described by Fernández-Vizarra et al. [10]. Heart mitochondria lipid peroxidation was quantified by measuring the thiobarbituric acid reactive substance, malondialdehyde (MLDA). An aliquot of heart mitochondria suspension (100 μL) was incubated with 185 μL of a solution containing acetic acid (Sigma-Aldrich) 50 % in water, 1.3 % thiobarbituric acid (Merck KGaA, Darmstadt, HES, Germany) and phosphate buffer solution, pH 7.2, in proportion 1:1.5:1.2, respectively, for 30 min at 90 °C. After that, the samples were centrifuged (9000*g* for 10 min at 4 °C) and the supernatants collected for measurement of absorbance at 532 nm using a multiplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). Malondialdehyde (Sigma-Aldrich) was used as a standard.

Quantification of serum creatine kinase

Blood samples of animals treated with high dose of mito-T were taken and centrifuged at 1200*g* for 20 min at 4 °C. Serum was collected and quantified for total creatine kinase (CK) and CK-MB using diagnostic kits (Gold Analisa Diagnóstica Ltda, Belo Horizonte, MG, Brazil), following the manufacturer's recommendations.

Statistical analysis

The normality of the data was assessed by the D'Agostino and Pearson normality test. Statistical differences were

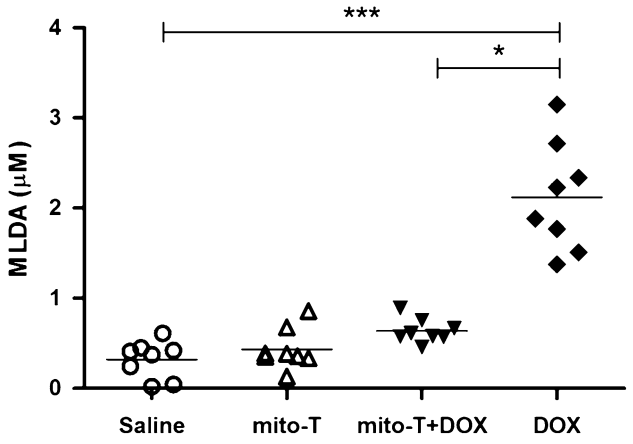

Fig. 2 Lipid peroxidation in heart mitochondria of mice treated with two mito-TEMPO (mito-T; 5 mg/kg body weight) doses and/or once with doxorubicin (DOX; 24 mg/kg body weight). Mice were treated intraperitoneally with mito-T, 48 and 24 h before administration of DOX. Heart mitochondria lipid peroxidation was quantified by measuring the malondialdehyde (MLDA). Negative control mice received only saline. *Each symbol* represents the result obtained from a single animal. *Horizontal lines* represent the median values for groups of eight animals. Comparisons among groups were performed by Dunn's multiple comparison test. *$p < 0.05$; ***$p < 0.001$

analyzed using Kruskal–Wallis test followed by Dunn's test. Statistical significance was accepted at $p \leq 0.05$. All analysis was performed using the GraphPad Prism 5.0 Software (San Diego, CA, USA).

Results

DOX induced lipid peroxidation in heart mitochondria ($p < 0.001$), and DOX-treated mice receiving mito-T at low dose showed level of MLDA significantly lower compared to DOX group ($p < 0.01$). Furthermore, administration of mito-T alone did not cause any significant change from control values (Fig. 2).

Additionally, the mice treated with mito-T at high dose showed decrease in serum levels of total CK compared to DOX alone ($p < 0.05$). Although not significantly, the levels of CK-MB were reduced compared to DOX-treated group (Fig. 3). No differences were seen between mito-T-injected and mito-T-untreated mice in the low-dose mito-T experiment (data not shown).

Discussion

The search to new substances and therapeutic strategies to attenuate chemotherapy toxicity continues. As the cardiac susceptibility to DOX-induced oxidative stress has been associated with the high oxidative metabolism of the heart and its lower level of antioxidant enzymes and with the affinity of DOX to cardiolipin [11, 12], in the present investigation it was evaluated whether mito-T pretreatment could produce cardioprotective effects in mice treated with DOX.

Pretreatment of animals with mito-T (5 mg/kg, 48 and 24 h prior to DOX) significantly reduced the lipid peroxidation of heart mitochondria ($p < 0.01$) indicating targeting of the antioxidant to the mitochondria, as referred by Dikalova et al. [6]. Lipid peroxidation is considered a good cardiotoxicity indicator for DOX [4]; therefore, their reduction indicates that mito-T probably acts as an antioxidant that could prevent the mitochondrial disfunction DOX induced and its consequently cardiotoxicity.

Increase in CK serum level after DOX administration is referred as a consequence of heart injury [13]. Pretreatment with mito-T (20 mg/kg) twice, 24 and 1 h before

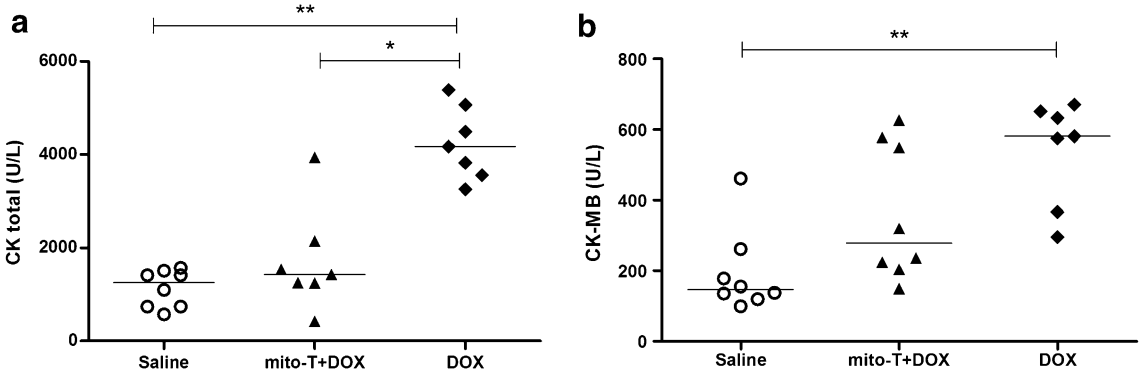

Fig. 3 Serum levels of creatine kinase (CK) total (**a**) and CK-MB (**b**) in heart of mice treated with two mito-TEMPO (mito-T; 20 mg/kg body weight) doses and/or once with doxorubicin (DOX; 24 mg/kg body weight). Mice were treated intraperitoneally with mito-T, 24 and 1 h before administration of DOX. *Each symbol* represents the result obtained from a single animal. *Horizontal lines* represent the median values for groups of seven or eight animals. Comparisons among groups were performed by Dunn's multiple comparison test. *$p < 0.05$; **$p < 0.01$

DOX treatment, inhibited the increase in CK total serum level ($p < 0.05$) induced by DOX. No significant differences were observed in CK-MB serum levels, however, probably because of great variation within groups. In work evaluating different doses of mito-T, Patil et al. [7] suggest that accumulation of higher dose of mito-T within the mitochondria could depolarize membrane and impair their protector effect. Thus, the therapeutic effects of this antioxidant at high dose should be better investigated.

Conclusion

Our data suggest that mito-T at a relatively high dose protects mice against DOX-induced cardiotoxicity; however, as the precise mechanism of the antioxidant activity of mito-T remains unclear, further investigations are required to confirm this assumption.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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