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Baicalein alleviates doxorubicin-induced cardiotoxicity via suppression of myocardial oxidative stress and apoptosis in mice

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ABSTRACT

Aims: Doxorubicin is a widely used anthracycline derivative anticancer drug. Unfortunately, the clinical use of doxorubicin has the serious drawback of cardiotoxicity. In this study, we investigated whether baicalein, a bioflavonoid, can prevent doxorubicin-induced cardiotoxicity in vivo and we delineated the possible underlying mechanisms.
Main methods: Male BALB/c mice were treated with either intraperitoneal doxorubicin (15 mg/kg divided into three equal doses for 15 days) and/or oral baicalein (25 and 50 mg/kg for 15 days). Serum markers of cardiac injury, histology of heart, parameters related to myocardial oxidative stress, apoptosis and inflammation were investigated.
Key findings: Treatment with baicalein reduced doxorubicin-induced elevation of serum creatine kinase-MB isoenzyme (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and ameliorated the histopathological damage. Baicalein restored the doxorubicin-induced decrease in both enzymatic and non-enzymatic myocardial antioxidants and increased the myocardial expression of nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1). Further studies showed that baicalein could inverse the Bax/Bcl-2 ratio, suppress doxorubicin-induced p53, cleaved caspase-3 and PARP expression and prevented doxorubicin-induced DNA damage. Baicalein treatment also interferes with doxorubicin-induced myocardial NF-κB signaling through inhibition of IκBα phosphorylation and nuclear translocation of p65 subunit. Doxorubicin elevated iNOS and nitrites levels were also significantly decreased in baicalein treated mice. However, we did not find any significant change ($p > 0.05$) in the myocardial TNF-α and IL-6 levels in control and treated animals.
Significance: Our finding suggests that baicalein might be a promising molecule for the prevention of doxorubicin-induced cardiotoxicity.

1. Introduction

Doxorubicin is an effective and widely used anthracycline derivative cytotoxic antibiotic. It is used for the treatment of a variety of neoplasm's including leukemias, lymphomas and solid human malignancies [26]. Despite being a highly targeted molecule for cancer therapy, the clinical use of doxorubicin is limited because of cardiotoxicity leading to dilated cardiomyopathy with congestive heart failure [18]. The mechanism involved in doxorubicin-induced cardiotoxicity is complex and multifactorial and is known to involve, at least in part, oxidative stress and mitochondrial dysfunction, which eventually lead to cardiomyocyte death by apoptosis and/or necrosis [27]. Cardiomyocytes are highly prone to reactive oxygen species (ROS)-induced damage because of lesser amount of antioxidant defense, higher density of mitochondria occupancy and high aerobic metabolism when compared to cells of other tissues [11]. It has also been demonstrated that doxorubicin-induced ROS generation activates NF-κB to elicit subsequent apoptosis in cardiomyocytes [8,10]. Moreover, generation of ROS promotes lipid peroxidation and apoptotic change due to loss of mitochondrial membrane integrity and p53 activity [31]. Although extensive efforts have been made to prevent doxorubicin-induced cardiotoxicity, there is little harmony and agreement in the best approach.

The health benefits of polyphenolic compounds of plant origin have been widely studied. Baicalein (5, 6, 7-trihydroxy flavone) is a major active constituent in the roots of medicinal herb *Scutellaria baicalensis* Georgi (Lamiaceae family) [33]. The root of *S. baicalensis* has been used as folk medicine in China for the treatment of inflammatory

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diseases, chronic hepatitis, bacterial and viral infections, allergy and thrombotic stroke [14]. Baicalein is also known to exhibit therapeutic effect in endotoxin-induced myocardial dysfunction [20] and ameliorates myocardial ischemic/reperfusion injury in rats [16,36]. It has also been reported that baicalein attenuates ROS generation and cell death during doxorubicin exposure and exhibits cytoprotective effect against doxorubicin-induced cardiomyocyte injury [4]. Although an in vitro study conducted by Chang et al. [4] addressed the beneficial effects of baicalein on doxorubicin-induced cardiomyocyte injury, there is no in vivo study conducted as yet to understand the mechanism of action and beneficial effect of baicalein on heart in doxorubicin-induced cardiotoxic animals. Hence, present study was designed to elucidate the molecular mechanisms underlying the protective effect of baicalein on heart using doxorubicin-induced cardiotoxicity model in mice.

2. Materials and methods

2.1. Chemicals, kits and antibodies

Baicalein (≥98.0% purity), doxorubicin, superoxide dismutase (SOD) assay kit, catalase, β-nicotinamide adenine dinucleotide 3-phosphate reduced form (NADPH), 2-thiobarbituric acid (TBA), 1-chloro-2, 4-dinitrobenzene (CDNB), 2, 6-dichlorophenolindophenol (DCIP), reduced glutathione (GSH), 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Co, St. Louis, MO, USA. Halt protease inhibitor cocktail, Bicinchoninic acid (BCA) protein assay kit, NE-PER nuclear and cytoplasmic extraction kits were purchased from Pierce Biotechnology, Rockford, IL, USA. Mouse TNF-α and IL-6 ELISA (Ready-SET-Go) kits were obtained from eBioscience, USA. All other chemicals were of analytical grade. Antibodies against Nrf2, HO-1, cleaved caspase-3, cleaved PARP, Bax, Bcl-2, p53, NF-κB (p65), IκBα, phospho-IκBα, β-actin, Lamin B and horseradish-peroxidase (HRP)-conjugated secondary antimouse and antirabbit antibodies were obtained from Cell Signaling Technology (Boston, MA). Antibody against iNOS was obtained from Sigma-Aldrich Co, St. Louis, MO, USA.

2.2. Animals

The animal experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of CSIR-Indian Institute of Chemical technology (IICT), Hyderabad, India (Approval No: IICT/PHARM/SRK/26/02/2014/05). Male BALB/c mice, 7–8 weeks age (25 to 27 g weight), were purchased from National Institute of Nutrition (NIN), Hyderabad, India and maintained in the housing facility of our institute under controlled environmental conditions with a 12/12 h light/dark cycle. The experiment was performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India for safe use and care of experimental animals. The mice were provided with rodent chow and water ad libitum. Animals were acclimatized for 7 days before initiation of experimentation.

2.3. Experimental design

Mice were randomly divided into five groups: the vehicle control group (Control, n = 8), received daily oral gavage of 2% gum acacia suspension for 15 days and an intraperitoneal injection of normal saline on 5th, 10th and 15th day; the doxorubicin control group (Dox, n = 8), received an intraperitoneal injection of 5 mg/kg doxorubicin dissolved in normal saline on 5th, 10th and 15th day to obtain a cumulative dose of 15 mg/kg over a period of 15 days; the baicalein (25 mg/kg) and doxorubicin treated group (Dox + Bai-25, n = 8), received daily oral gavage of 25 mg/kg baicalein suspension in 2% gum acacia for 15 consecutive days and an intraperitoneal injection of 5 mg/kg doxorubicin dissolved in normal saline on 5th, 10th and 15th day to obtain a cumulative dose of 15 mg/kg; the baicalein (50 mg/kg) and doxorubicin treated group (Dox + Bai-50, n = 8), received daily oral gavage of 50 mg/kg baicalein suspension in 2% gum acacia for 15 consecutive days and an intraperitoneal injection of 5 mg/kg doxorubicin dissolved in normal saline on 5th, 10th and 15th day to obtain a cumulative dose of 15 mg/kg; the baicalein control group (Bai, n = 8), received daily oral gavage of 50 mg/kg baicalein suspension in 2% gum acacia for 15 consecutive days and an intraperitoneal injection of normal saline on 5th, 10th and 15th day. The dose of the baicalein was selected based on the previous studies, in which it showed significant protection in liver against carbon tetrachloride-induced liver fibrosis [37] and on heart, against coronary artery ligation-induced myocardial infarction in rats [16]. The dose and duration of doxorubicin treatment was selected based on the previous report with slight modification [2].

After 24 h of last dose of doxorubicin (i. e. on 16th day), body weight of all experimental animals was recorded, blood samples were collected through retro-orbital plexus and euthanized under CO_2 asphyxiation. Heart tissue was collected, weighed, frozen in liquid nitrogen to stop metabolic activity and was stored in −80 °C till further analysis. Relative weight of heart tissue was calculated to assess the effect of baicalein on doxorubicin-induced changes in cardiac tissue mass.

2.4. Assessment of serum markers of cardiac injury

For preparation of serum, blood samples were kept at room temperature for 30 min to clot and then, centrifuged at 4000 rpm for 15 min. Supernatant was collected and used for estimation of serum specific cardiac injury biomarkers such as creatine kinase-MB isoenzyme (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities by using respective commercial kits (Siemens, India) and auto blood analyzer (Siemens, Dimension Xpand^{plus}, USA).

2.5. Histopathological examination

Heart tissue samples were fixed in 10% neutral buffered formalin for 48 h at room temperature, dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded in paraffin. Approximately 5 μm thick sections were prepared from tissue paraffin block and stained with hematoxylin and eosin (H & E). The slides were evaluated for any structural changes under light microscope (Zeiss microscope, Axioplan 2 Imaging, Axiovision software).

2.6. Assessment of myocardial markers of oxidative stress

Frozen heart tissue samples were homogenized in ice-cold phosphate buffer saline (50 mM, pH 7.4) using Teflon homogenizer to obtain a 12% (*w*/*v*) heart tissue homogenate. The homogenates were centrifuged at 12,000 rpm for 30 min at 4 °C to obtain a clear supernatant. The supernatant was pooled and used for measurement of activities of antioxidant enzymes, superoxide dismutase (SOD) (Sigma-Aldrich Co, St. Louis, MO, USA), catalase (CAT) [1], glutathione S-transferase (GST) [13], NAD(P)H: quinone oxidoreductase 1 (NQO1) [45] and tissue levels of reduced glutathione (GSH) [9], vitamin C [30], thiobarbituric acid reactive substance (TBARS) [29] and nitrites [32] by spectrophotometrically as described previously.

2.7. DNA isolation and fragmentation assay

DNA was isolated from heart tissue using proteinase k digestion and phenol/chloroform/isoamyl alcohol (25:24:1, *v*/*v*/v, pH 8.0) extraction method as described in our earlier literature [34]. To detect DNA fragmentation, extracted DNA was electrophoretically separated on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and visualized and photographed under UV light (Bio Doc-It, Imaging system).