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Original Article

Protective effect of *Rheum turkestanikum* root against doxorubicin-induced toxicity in H9c2 cells

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ABSTRACT

Doxorubicin is a chemotherapy drug but its clinical using is limited because of its cardiotoxicity. Reactive oxygen species play an important role in the pathological process. The aim of this study is to evaluate the protective effect of *Rheum turkestanicum* Janisch., Polygonaceae, against doxorubicin-induced apoptosis and death in H9c2 cells. The cells were incubated with different concentrations of *R. turkestanicum* extract and *N*-acetylcysteine as positive control for 2 h, followed by incubation with 5 μM doxorubicin for 24 h. Cell viability and apoptotic induction were determined by using MTT and PI assays, respectively. The level of reactive oxygen species and lipid peroxidation was measured by fluorimetric methods. Doxorubicin significantly decreased cell viability which was accompanied by an increase in ROS production and lipid peroxidation. Pretreatment with *R. turkestanicum* increased the viability of cardiomyocytes and could decrease lipid peroxidation and reactive oxygen species generation. Also, *R. turkestanicum* attenuated apoptotic induction. *N*-acetylcysteine at 100 μM reduced the levels of reactive oxygen species and lipid peroxidation. But, treating H9c2 cells with *N*-acetylcysteine did little to protect H9c2 cells from doxorubicin-induced cell death. *R. turkestanicum* exerts protective effect against oxidative stress-induced cardiomyocytes damage. Our findings showed that *R. turkestanicum* could exert the cardioprotective effects against doxorubicin-induced toxicity partly by anti-apoptotic activity. Also, *N*-acetylcysteine prevented oxidative stress via reduction of reactive oxygen species and lipid peroxidation. *N*-acetylcysteine induced less protective effects than *R. turkestanicum* extract against doxorubicin-induced cytotoxicity.

Introduction

Doxorubicin (DOX) as an effective chemotherapy drug has serious side effects such as dilated cardiomyopathy and congestive heart failure (Turakhia et al., 2007). Therefore, using this drug must be limited. The mechanisms of DOX-induced cardiotoxicity are not completely understood, but most evidences indicate that the generation of reactive oxygen species (ROS) is involved (Bryant et al., 2007). Increased level of ROS leads to protein and lipid peroxidation, DNA damage and irreversible cell damage (Ghorbani et al., 2015; Asadpour et al., 2014). ROS can directly impair contractile function, activate hypertrophy signaling pathways, stimulate cardiac fibroblast proliferation and induce extracellular matrix remodeling (Takimoto and Kass, 2007; Tsutsui et al., 2011). Excessive ROS also cause accumulation of intracellular Ca^{2+} in cardiac cells which increases the mitochondrial permeability and leads to the release of cytochrome c into the cytoplasm and the following apoptotic cascades (Pacher et al., 2001). Interestingly, some natural foods have been reported to contain substantial amounts of antioxidants and free radical scavenging agents. These compounds diminish some side effects of chemotherapeutic agents on normal cells by reducing their genotoxicity (Bryant et al., 2007). H9c2 myoblasts, a rat embryonic cell line, which has the ability to differentiate between a skeletal or cardiac muscle phenotype, can be instrumental in understanding DOX cytotoxicity in different stages (Branco et al., 2012). The recent studies have shown that some of the herbs act against oxidative injury-related cardiotoxicity. *Rheum* species, Polygonaceae, have a long history as medicinal plants in traditional Chinese medicine. The main active ingredients of the *Rheum* species are a series of anthraquinones, dianthrones, glycosides and tannins. The anthraquinone derivatives include emodin, rhein, chrysophanol, physcion, alizarin, citreorosein, and aloe-emodin (Dorsey and Kao, 2007). *R. turkestanicum* Janisch. is a plant that grows widely in central Asia and also in northeast of Iran. Traditionally, people use roots of *R. turkestanicum* as an antidiabetic and anti-hypertensive as well as anticancer agent

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(Dorsey and Kao, 2007). Recent studies have shown other species of *Rheum* such as *R. undulatum* is containing anti-oxidant compounds. Rhapontigenin and rhaponticin are isolated from *R. undulatum* scavenge ROS, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and hydrogen peroxide (H_2O_2) (Zhang et al., 2007). Also, these compounds decrease membrane lipid peroxidation and cellular DNA damage, which are the main targets of oxidative stress-induced cellular damage (Zhang et al., 2007). The recent study showed some of isolated anti-oxidant compounds from Rheum species such as *R. emodi* protected H9c2 cells against H_2O_2 (Chai et al., 2012). In this study, the protective effect of hydro-alcoholic extract of *R. turkestanicum* on DOX-induced cardiotoxicity was evaluated for first time.

Material and methods

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), thiobarbituric acid (TBA), 2,7-dichlorofluorescin diacetate (DCFH-DA), propidium iodide (PI), *N*-acetylcysteine (NAC), sodium citrate and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco's Modified Eagles Medium (DMEM), penicillin-streptomycin and fetal bovine serum were purchased from Gibco. Trichloro acetic acid (TCA) and malondialdehyde bis-(dimethyl acetal) (MDA) were obtained from Merck (Darmstadt, Germany).

Preparation of extracts

The root of *Rheum turkestanicum* Janisch., Polygonaceae, was collected from Chenar, a village in Zavin Rural District, Kalat County, Razavi Khorasan Province, Iran. The plant was identified by M.R. Joharchi, from Ferdowsi University of Mashhad Herbarium. Voucher specimen (No. 21377) was deposited in Ferdowsi University of Mashhad Herbarium. Dried *R. turkestanicum* root (20 g × 3) was ground into fine powder and then 50 g of this powder was subjected to extraction with 70% ethanol in a Soxhlet apparatus for 48 h. The hydro-alcoholic extract was then dried on a water bath and kept frozen in less than −18 °C for the following use. The yield of extract was 19% (w/w). The extract was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 50 mg/ml before being used in cytotoxicity and apoptosis assays.

Cell culture

Rat heart cell line H9c2 was obtained from American Type Culture Collection (ATCC CRL-1446) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin. For the experiments, they were seeded in 96-well and 24-well culture plates for MTT/ROS and MDA assays, respectively. For apoptosis assay, cells were seeded at 100,000/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with extract (6–200 μg/ml) for 2 h and then incubation was continued in the presence of the extract with 5 μM doxorubicin for 24 h. Also, NAC was applied as positive control at 10 and 100 μM.

Cell viability

The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay as described previously (Mosmann, 1983; Malich et al., 1997). Briefly, MTT solution in phosphate-buffered saline (5 mg/ml), was added to each well at final concentration of 0.05%. After 3 h, the formazan precipitate was dissolved in DMSO. The absorbance at 570 and 620 nm (background) was measured using a StatFAX303 plate reader. The experiment was carried out in triplicate.

Lipid peroxidation assay

The level of lipid peroxidation was estimated by measuring MDA which is the end product of lipid peroxidation. At the end of incubation, the cells were scraped and centrifuged at 13,000 × *g* at 4 °C for 30 min (Buege and Aust, 1978). Then, 400 μl of TCA (15%) and 800 μl of TBA (0.7%) were added to 500 μl of cell samples. The mixture was vortexed and then heated for 40 min in a boiling water bath. Then, 200 μl of the sample was transferred to 96-well plate and the fluorescence intensity was read with excitation/emission of 480/530 nm. The experiment was carried out in triplicate.

Measurement of reactive oxygen species

Intracellular ROS level was evaluated using a fluorescent probe, DCF-DA (Wu and Yotnda, 2011). At the end of incubation, the cells were treated (30 min) with DCFH-DA (10 μM) at 4 °C in the dark. Then, the fluorescence intensity was detected with excitation/emission of 485/530 nm. The experiment was performed in triplicate.

Apoptosis

Apoptotic cells were detected by using PI staining of the treated cells followed by flow cytometry to detect the so-called sub-G1 peak. Briefly, H9c2 cells were cultured overnight in a 24-well plate and pretreated by *R. turkestanicum* for 4 h and then treated with DOX for 24 h. Floating and adherent cells were then harvested and incubated at 4 °C overnight in the dark with 500 μl of a hypotonic buffer (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100). The experiment was carried out in triplicate (Nicoletti et al., 1991).

Statistics

All data were expressed as mean ± SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tamhane's T2 post hoc test. Differences were considered significant at $p < 0.05$.

Results

Effect of DOX on cell viability

Incubation of the cells with different concentrations of DOX (2.5–20 μM) showed that the cell viability reduced in a dose dependent manner (Fig. 1).

Effect of R. turkestanicum *extract on cell viability*

Incubation with 6 μM DOX significantly decreased cell viability to 55 ± 4.5% of control ($p < 0.001$). Pretreatment with 12–200 μg/ml of *R. turkestanicum* could increase the viability of H9c2 cells to 71 ± 0.68% ($p < 0.05$), 74.6 ± 2.5% ($p < 0.01$), 79 ± 1.3% ($p < 0.001$), 84 ± 3.6% ($p < 0.001$) and 88 ± 1.3% ($p < 0.001$) of control, respectively (Fig. 2). However, at dose of 6 μg/ml, *R. turkestanicum* was not able to protect H9c2 cells against DOX-induced cytotoxicity. Also pretreatment of cells with NAC at doses of 10 and 100 μM, protected cells against DOX (5 μM) at dose of 100 μM (69 ± 1.8%, $p < 0.05$) (Fig. 2).