Danshen–Gegen decoction exerts proliferative effect on rat cardiac myoblasts H9c2 via MAPK and insulin pathways

Chi Chun Fong [a,b], Fan Wei [a], Yao Chen [a,b], Wai Kin Yu [a], Chi Man Koon [c,d], Ping Chung Leung [c,d], Kwok Pui Fung [c,d,e], Clara Bik San Lau [c,d], Mengsu Yang [a,b,*]

[a] Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong
[b] Shenzhen Key Laboratory of Biochip Research, City University of Hong Kong, Shenzhen 518057, PR China
[c] Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong
[d] State Key Laboratory of Phytochemistry & Plant Resources in West China, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong
[e] School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

ARTICLE INFO

ABSTRACT

Ethnopharmacological relevance: Danshen (root of *Salvia miltiorrhiza*) and Gegen (roots of *Pueraria lobata*) are traditional Chinese medicines that have been used in combination for cardiovascular disease treatment.

Aim of the study: The present study was performed to investigate the effect of Danshen–Gegen decoction on rat myocardium cell line H9c2 and the possible molecular mechanisms.

Materials and methods: Rat heart myocardium H9c2 cells were treated with or without Danshen–Gegen decoction (DG) ranging from 10 to 1000 µg/ml for 24 h. Cell viability was measured by Alarma blue assay and cell proliferation assay was performed by BrdU Cell Proliferation ELISA kit. The activation of mitogen-activated protein kinase and insulin pathways was analyzed by Luminex technology and the growth factors and cytokine expression of H9c2 cells induced by DG was evaluated by protein array. Moreover, a rat functional specific cDNA microarray was constructed to study the gene expression profiles of H9c2 cells upon the DG treatment at 50 µg/ml for 24 h.

Results: DG promoted H9c2 cell viability and cell proliferation at dose-dependent manner within the range between 0 and 250 µg/ml. A Bio-Plex assay kit (Bio-Rad Bioscience) was used to detect the expression level of phosphoprotein as well as total proteins involved in the MAPK and insulin pathways. Significant phosphorylation of ERK, c-Jun, JNK, p38, AKT, IGF-IR, IRS-1 and I kappa B were observed after DG treatment at 2 h or 4 h. A rat cytokine antibody array was used to detect and quantify 22 growth factors and cytokines in samples collected from the control and DG treated H9c2 cells. In the category of growth factors, GM-CSF, CNIF and b-NGF were stimulated by DG, while the expression of TIMP-1 was suppressed. For cytokine expression, it was found that DG stimulated three interleukin subclasses, IL-1α, 1X and 6, respectively. However, the expression of pro-inflammatory factors such as TNF-α and IFN-γ were down-regulated significantly. Moreover, the microarray analysis revealed that DG significantly up-regulated anti-apoptosis related genes such as Cdkn2c and Ppp3ca, and several cardiovascular disease suppressers and anti-inflammatory mediators; on the other hand, pro-apoptotic related genes including Caspase and Tnf-α were down-regulated by DG. Based on the results, a tentative scheme was proposed to show that the activation of the MAPK and insulin pathways are involved in the bioactive effect of Danshen–Gegen decoction on cardiomyocytes.

Conclusion: Our study suggested that Danshen–Gegen decoction has proliferative effect on myocardium cells via MAPK and insulin signaling pathways. The molecular mechanism of the action may include the up-regulation of IRS/AKT and JNK pathways as well as the inhibition of TNF and p38 pathways.

1. Introduction

Cardiovascular diseases refer to a class of diseases that involve the heart or blood vessels. The most common forms of heart diseases include coronary artery disease, heart attack, heart failure, high blood pressure and stroke. Both Danshen and Gegen are well known for their putative cardio protective and

* Corresponding author at: Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong. Tel.: +852 34427797; fax: +852 34420552.
E-mail address: bhmyang@cityu.edu.hk (M. Yang).

anti-atherosclerotic effects in traditional Chinese medicine, and their antioxidant properties are thought to be the common reason for their pharmacological use in heart ischemia (Cheng, 2006; Xu et al., 2007). Generally, Gegen (root of *Pueraria lobata*) comes from the kudzu root and protects the myocardium against ischemia injury via inhibiting mitochondrial permeability, blocking cardiac ion channels, and opening the collateral circulation (Wu et al., 2007). Danshen (root of *Salvia miltiorrhizae*) inhibits mitochondrial membrane permeability transmission by reducing protein thiol oxidation and exhibits properties of improving microcirculation, increasing coronary blood flow, suppressing thromboxane formation, inhibiting platelet adhesion and aggregation, and protecting against myocardial ischemia (Zhou et al., 2005; Cheng, 2007). Both *in vivo* and *in vitro* studies demonstrated that Danshen and Gegen formula showed the ability to modulate key early events in atherosclerosis and protect against isoprotereno-induced myocardial injury, and serve as a useful cerebroprotective agent (Sieveking et al., 2005; Lam et al., 2010; Wong et al., 2011). In recent years, a clinical research of Danshen and Gegen formulation (DG, ratio 7:3) has been carried out and its cardioprotective effects has been proven (Worldwide clinical trial listing, 2008). However, the molecular mechanism behind the effect has not been well documented. In the present study, we used rat myocardium cells H9c2 treated with Danshen–Gegen decoction (7:3) as a cellular model to investigate the molecular mechanism of the action of the decoction, particularly the involvement of the MAPK and insulin pathways that have been implicated in several diseases with increased cardiovascular risk and mortality (Morisco et al., 2006; Yin et al., 2008).

In this study, the activation of several signaling molecules involved in the MAPK and insulin pathways, such as ERK, c-Jun, JNK, p38, AKT, IGF-IR, IRS-1, and I kappa-B, in rat myocardial cells treated by Danshen–Gegen decoction were investigated using Luminex technology. The expression levels of growth factors and cytokine were determined by protein array technology. Moreover, gene array technology was used to identify transcriptional profiles of rat heart myocardium cells upon the treatment of Danshen–Gegen decoction. The effects of Danshen–Genen decoction on the myocardium were determined and the molecular pathways involved were proposed through analysis of the biological and molecular functions.

2. Materials and methods

2.1. Herbal materials preparation for treatment

Danshen–Gegen extract (7:3, w/w) (DG) was provided by the Institute of Chinese Medicine (ICM), Chinese University of Hong Kong. The extract has an optimized ratio as assessed by cardioprotection against ischemia/reperfusion injury, and was prepared in large scale for experimental and clinical investigations (Chiu et al., 2011). Voucher specimens of Radix Salviae Miltiorrhiza (#2008-3166a) and Radix Puerariae Lobatae (#2008-3167a) were deposited in the ICM. Danshen and Gegen were mixed in the ration of 7:3 and soaked in water (1:10, w/v) for 1.5 h, followed by extraction in boiling water (100 °C) for 60 min. The extraction procedure was repeated twice with boiling water (1:8, w/v) for 60 min and 30 min. The pooled aqueous extracts were concentrated under reduced pressure at 65 °C and the concentrate was dried to obtain the powdered form of DG extract with a yield of 10.1%.

The identification and quantification of chemical markers in DG extract was previously done by using HPLC (Lam et al., 2010), and the contents of seven quantifiable components, namely danshensu, protocatechuic aldehyde, puerarin, daidzein 8-Capiosyl-glucoside, daidzin, salvianolic acid B and daidzein in DG water extract were reported.

For the treatment, DG extract was freshly prepared for each experiment by dissolving in serum-free DMEM medium and sterilized by syringe filter (0.22 μm).

2.2. Cell viability assay

Rat cardiac myoblasts cell line H9c2 was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were maintained in complete DMEM with 10% FBS as well as 1% of Penicillin–Streptomycin (Gibco, USA) in a humidified 5% CO_2 incubator at 37 °C.

For cell viability assay, 100 μl (2×10^4 cells per ml) of H9c2 cell suspension in complete DMEM medium was seeded to each well of the 96-well tissue culture plate for overnight, then the culture medium was removed and cells were exposed to different dosage of Danshen–Gegen extract in serum-free DMEM medium (0–1000 μg/ml) for 24 h. Cells were then washed with $1 \times$ PBS twice and exposed to 100 μl of resazurin solution ($1 \times$) diluted with the medium for 5–6 h. Fluorescence signals were measured at 530 nm of excitation and 590 nm of emission. Experiments were performed in triplicate using different batches of cells and data were presented as means ± S.D.

2.3. Cell proliferation assay

To investigate the effect of DG extract on H9c2 cells proliferation, 2000 cells were seeded in 96-well plates for overnight in complete culture medium, then the culture medium was removed and the cells were exposed to different dosage of DG, 0, 10, 25, 50, 100 and 250 μg/ml, respectively. The treatments lasted for 24 h at 37 °C, and Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche Diagnostics, USA) was used to measure the proliferating cells according to the manufacturer's instructions. The optimized dosage of Danshen–Gegen decoction was used for other experiments.

2.4. Cell cycle analysis

The H9c2 cells were treated with 50 μg/ml of DG for 24 h; untreated cells were used as control. Before harvest, the cells were washed one time with phosphate-buffer saline (PBS) and re-suspended in 1 ml of hypotonic propidium iodide (Sigma) solution (50 μg/ml) containing 0.1% sodium citrate, 0.1% Triton X-100 and 100 mg/ml DNase-free RNase A for DNA staining. Cell cycle was measured by ESP flow cytometry (FACSCablibur Flow Cytometer, Becton Dickson, USA) at excitation 488 nm/emission 600 nm. Data were analyzed using cycle distribution software (ModFit LT version 2.0, Verity Software House, USA).

2.5. Analysis of MAPK and insulin pathway activation using Luminex technology

The phorphorylation states of insulin and MAPK signaling pathway-related molecules, e.g. ERK, c-Jun, JNK, p38, AKT, IGF-IR, IRS-1 and I kappa B, in H9c2 cells treated by DG were analyzed, by using Bio-Plex phosphoproteins and total target assay (Bio-Rad). Briefly, H9c2 cells were treated with DG at 50 μg/ml and the cell lysates were collected at 2 and 4 h after treatment, respectively. The phosphoproteins and total protein of MAPK and insulin pathway related molecules were analyzed with Bio-Plex 200. Data were presented as the fold change of fluorescence intensity in DG treated cells compared to the control cells.

2.6. Cytokine antibody array

A rat cytokine antibody array (Raybiotech Inc.) was used to simultaneously detect and quantify 22 cytokines in samples