Original Article

Study on Esculetin to reduce cardiotoxicity induced by Doxorubicin

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Abstract.

Objective: To investigate the protective effects and mechanisms of esculetin(Esc) on H9C2 cells injury induced by doxorubicin (DOX).

Methods: H9C2 cells were cultured and divided into control group, model (DOX) group and intervention (ESC + DOX) group. Flow cytometry was used to detect apoptosis of H9C2 cells and reactive oxygen (ROS) level in H9C2 cells; Western blotting to detect the cleaved Caspase-3, cleaved PARP, bid and Bcl-2 protein expression in H9C2 cells.

Results: The number of apoptosis and ROS level of H9C2 cells in the model group, the expression of cleaved Caspase-3, cleaved PARP and Bid protein in the model group were obviously higher than control group; and the Bcl-2 protein expression was obviously lower ($P < 0.05$).The number of apoptosis and ROS level, cleaved Caspase-3, cleaved PARP and Bid protein expression in H9C2 cells in intervention group were obviously lower than model group;the Bcl-2 protein expression was obviously higher ($P< 0.05$).

Conclusion: Esculetin can reduce the cardiotoxicity induced by doxorubicin by reducing apoptosis and ROS level.

Key words: doxorubicin, esculetin, Apoptosis

Introduction

Doxorubic in (DOX) is an anthracyc line antibiotic with strong anti-tumor effect,which is widely used as a potent antitumour drug, examples like breast cancer, lymphoma and melanoma. But it can cause serious irreversible cardiomyopathy and congestive heart failure. Therefore, the clinical application of doxorubicin is limited.[1] The mechanism of doxorubicin induced cardiotoxicity is complex, including stimulating free radical formation, mitochondrial damage, apoptosis and so on.[2] Some studies have found that doxorubicin can cause endoplasmic reticulum stress, increase the production of reactive oxygen species (ROS), and then lead to apoptosis of cardiomyocyte[3]. Esculetin (Esc) is the main active component of the traditional Chinese medicine Qinpi. Chemically speaking, Esculetin is a hydroxycoumarin that is umbelliferone in which the hydrogen at position 6 is substituted by a hydroxy group. It is used in filters for absorption of ultraviolet light. It has a role as an antioxidant, an ultraviolet filter and a plant metabolite. In Chinese Medicine, it has has been shown that Esc not only has antitussive, expectorant and anti-asthmatic effects, but also has many physiological functions, such as anti-inflammatory, antibacterial, anti-oxidative, liver-protective, antitumor.[4] Studies have shown that Esc can scavenge oxygen free radicals and protect cells from damage caused by oxitussive, expectorant and antiasthmatic effects[5-6]. It is speculated that Esc can protect cardiomyocytes from DOX induced injury. Therefore, a rat embryonic cardiomyocyte (H9C2 cells) injury model induced by doxorubicin was established to explore the possible mechanism of Esc in reducing the cardiotoxicity induced by doxorubicin.

Materials and methods

Material: Rat embryonic cardiomyocytes H9C2 were sourced from the cell bank of Chinese Academy of Sciences. RPMI-1640 medium and fetal bovine serum (FBS) both from hyclone

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laboratories (USA); Lipofectamine rnaimax BCA protein concentration determination kit and av-pi detection kit both from beyotime biotechnology company (Nantong, China); Cleaved Caspase-3, cleaved PARP, Bid and Bcl-2 antibodies all from Proteintech (Wuhan, China); DCFH-DA from Invitrogen (USA); Doxorubicin and Aesculetin both from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

Cell culture: H9C2 cells were cultured in RPMI-1640 containing 10% FBS in an incubator based on 37^0 and 5% CO2. When the cells covered 80% of the bottom of the culture bottle, trypsin digestion was used to prepare single cell suspension. Then the cells were inoculated into the culture plate for grouping experiment.

MTT assay for the detection of H9C2 cells viability: H9C2 cells were inoculated into 96 well plates and the cell concentration was adjusted to 5×104 cells/ml, divided into three groups, control group and 5μM Esc group and 10μM Esc group. The cells in the control group were only added with culture medium, and the cells in the two Esc groups were added with 5μM, 10μM Esc culture for 2 hours; Then cells in each group were added with different concentrations of doxorubicin (0μM, 1μM, 2μM, 4μM, 8μM, 16μM) continue to culture for 48 hours. The culture medium was discarded and washed with PBS for three times, and then MTT working solution with a concentration of 5mg /ml was added to each well; After 4 hours, 100 μL dimethyl methylene oxide (DMSO) was added and the solutionwas shaken for 10 minutes. The absorbance value (OD) was measured at 570nm by microplate reader, and the cell survival rate was calculated. The cell survival rate (%) = (OD of administration group / OD of blank group)×100%.

Analysis of apoptosis: H9C2 cells were inoculated into 6 well plates and the cell concentration was adjusted to 1 × 106cells/ml. The cells were divided into three groups: control group, model group (DOX) and intervention group (Esc + DOX), with 3 multiple pores in each group. The cells in the control group were only added with culture medium; Model group cells were added with 8μM doxorubicin was cultured for 48 hours; The cells in the intervention group were added with 10μM Esc was cultured for 2h, and then 8μM doxorubicin was added ,culturing for 48 hours. Then the cells were washed with PBS and digested with trypsin. The collected cells were resuspended in 0.3ml binding buffer and added with 5μL AV and 5μL PI was incubated in the dark at room temperature for 15 minutes, then 0.2ml binding buffer was added for apoptosis detection.

Intracellular reactive oxygen species assay: 10μM DCFH-DA staining solution was added to each group of cells and incubated at $37^0$for 20 minutes. The total amount of ROS in each group of cells was detected by flow cytometry.

Western blot analysis of protein expression: The cells were collected, washed twice with PBS, added Ripa cell lysate and centrifuged at high speed for 5 minutes. The supernatant was collected, and the protein concentration was determined by BCA protein concentration determination kit, and protein electrophoresis was performed. After electrophoresis, the primary antibodies of cleaved Caspase-3, cleaved PARP, bid and Bcl-2 were added and incubated overnight at 4^0, and then the secondary antibodies were added for 2h at room temperature. They were developed and scanned by ECL method in dark room.

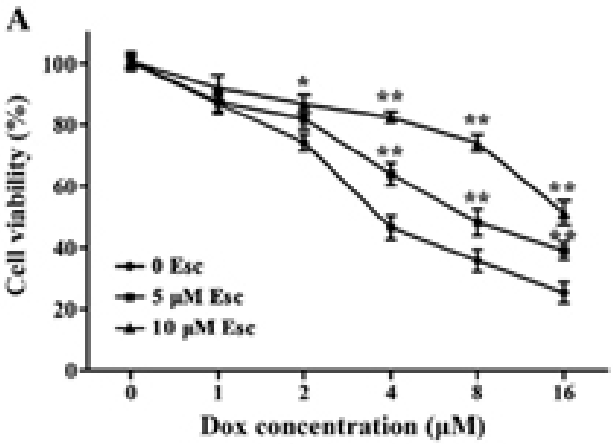
Statistical analysis: All experimental data are presented as mean ± standard deviation and analyzed by one-way ANOVA. A P-value <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS version 19.0.

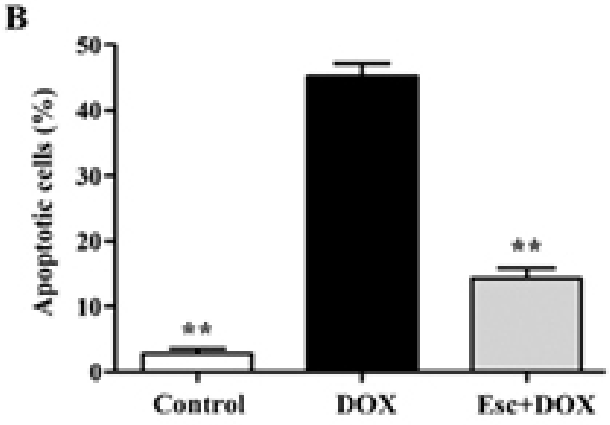
Results

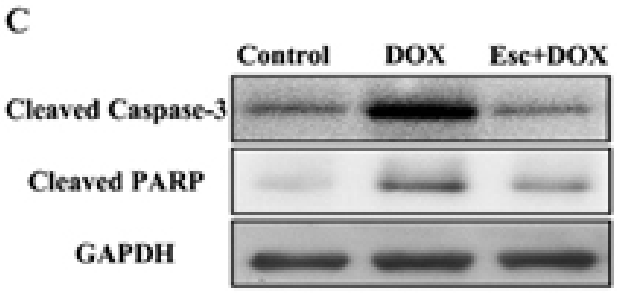
Survival rate of H9C2 cells: MTT results showed that with the increase of doxorubicin concentration, the survival rate of H9C2 cells in the control group decreased gradually in a concentration dependent manner ($P< 0.05$). Compared with the control group, the survival rate of H9C2 cells,pretreated with 5μM and 10μM Esc for 2 hours ,increased obviously ($P < 0.05$); And the effect of 10 μM Esc was better than 5μM ($P < 0.05$) (Fig. A).

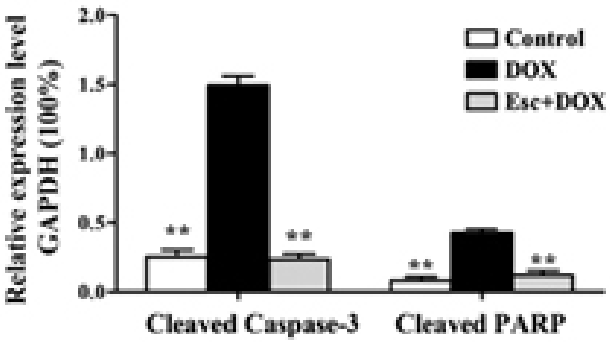
Apoptosis and ROS content of H9C2 cells: The apoptosis number and ROS content of H9C2 cells in the DOX group were obviously higher than control group, and the apoptosis number and ROS content of H9C2 cells in the Esc +DOX group were obviously lower than DOX group (P<0.05) (Fig. B).

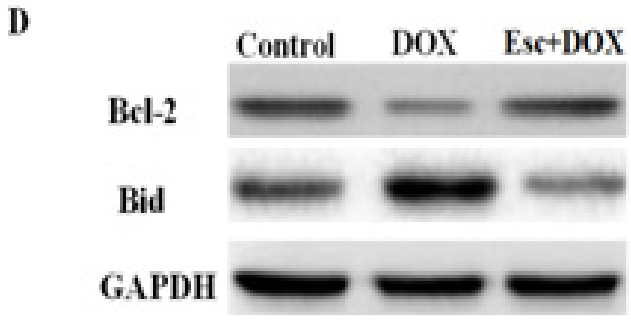
Expression of cleaved Caspase-3, cleaved PARP, bid and Bcl-2 proteins in H9C2 cells: The expression of cleaved Caspase-3, cleaved PARP and Bid protein in H9C2 cells in the Esc+DOX group was significantly lower than DOX group, and the expression of Bcl-2 protein was significantly higher than DOX group (P<0.05) (Fig. C,D).

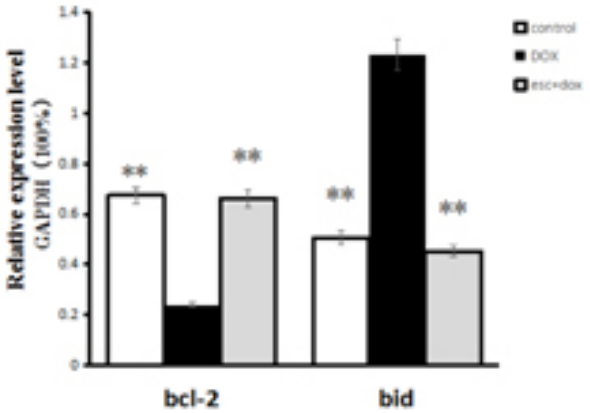












Discussion

Doxorubicin (DOX) is an anthracycline drug widely used in malignant tumor chemotherapy. It has remarkable therapeutic effect. However, doxorubicin has obvious cardiotoxicity, which seriously restricts its clinical application. Reducing the cardiotoxicity of doxorubicin during chemotherapy as much as possible has always been a concern of clinicians.

The mechanism of doxorubicin induced cardiotoxicity is complex, including increased production of intracellular reactive oxygen species, mitochondrial dysfunction, DNA damage, apoptosis and so on [7] .This study also confirmed the above results. The number of apoptosis and ROS content of H9C2 cells in the model group added with doxorubicin were significantly higher than those in the control group; At the same time, this study found that the expression of cleaved Caspase-3, cleaved PARP and Bid in H9C2 cells in the model group increased significantly, and the expression of Bcl-2 decreased significantly. It has been proved that apoptosis includes mitochondrial pathway, death receptor pathway and endoplasmic reticulum pathway. Caspase-3 is the most critical protease in the process of apoptosis. It is the common downstream effector of multiple death receptor-mediated apoptosis pathways. After caspase-3 is activated, it can enzymolysis and cleave specific substrates, such as DNA dependent protein kinase and sterol regulatory element binding protein, and cause cell apoptosis by changing its structure or affecting specific signal molecules.8 Cleaved PARP is the hydrolysate of activated caspase-3 in apoptotic cells and a marker of apoptosis. Bid and Bcl-2 are representative members of Bcl-2 family. Bid protein can promote apoptosis, and Bcl-2 can inhibit apoptosis.[9]

The results show that the number of apoptosis, the content of ROS and the expression of cleaved Caspase-3, cleaved PARP and Bid protein decrease significantly, and the expression of Bcl-2 protein increases significantly in the intervention group. This study suggests that esculetin can reduce apoptosis by regulating the expression of apoptosis related proteins such as cleaved Caspase-3, cleaved PARP, Bid and Bcl-2, and reduce the toxicity of doxorubicin to cardiomyocytes by reducing the production of reactive oxygen species. This study can provide experimental basis for relevant clinical studies, but other mechanisms of esculetin in reducing doxorubicin induced cardiotoxicity need to be further explored.

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