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Full Length Research Paper

Effect of catalpol on doxorubicin-induced cytotoxicity in H9c2 cells

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The aim of the present study was to investigate the protective effect of catalpol, an effective component of *Rehmannia glutinosa* Libosch, against doxorubicin (DOX)-induced cytotoxicity in H9c2 cells and to find out its potential mechanisms. The cell viability was assayed by MTT assay. Apoptotic cells were evaluated by Hoechst 33258 staining. The lactate dehydrogenase (LDH) leakage, the malondialdehyde (MDA) activity and intracellular of reactive oxygen species (ROS) levels were examined. The activity of antioxidant enzymes, including catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured. Furthermore, we evaluated the effect of catalpol on the antitumor activity of DOX in HeLa cervical carcinoma cells with MTT assay. The results of MTT assay and measurement of LDH release showed that catalpol significantly reduced DOX-induced damage. Catalpol reduced intracellular ROS, decreased the concentration of MDA and increased the activities of antioxidant enzymes in DOX-treated H9c2 cells. Importantly, the MTT analysis showed that the addition of catalpol did not interfere with the antitumor activity of DOX in HeLa cells. Taken together, our findings indicate that catalpol could exert the cardioprotective effects against DOX-induced toxicity without affecting its antitumor activity of this anthracycline.

Key words: Catalpol, doxorubicin, H9c2 cells, reactive oxygen species.

INTRODUCTION

Doxorubicin (DOX), an effective anticancer drug, is used to treat a variety of malignacies, such as leukemias, Hodgkin and non-Hodgkin lymphoma, and solid tumors (Young et al., 1981; Hortobagyi, 1997). Its clinical use is limited because of its dose-dependent cardiotoxicity. The mechanisms of DOX-induced cardiotoxicity are not completely understood, but a number of reports have indicated that the mechanism involves the generation of reactive oxygen species (ROS) (Takemura and Fujiwara, 2007). ROS can damage all components of the cardiomyocyte, including lipid, DNA and protein, and consequently result in the apoptosis or death of cell (Arola et al., 2000; Menna et al., 2007; Kumar et al., 2001; Wu et al., 2002). Antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), are an important antioxidant defense in cells. They are the specific oxygen free radical scavengers and can protect the cell against ROS-induced damage (Khalili et al., 2011). Catalpol is an effective component extracted from *Rehmannia glutinosa Libosch*, it has many pharmacological actions, and such as antioxidant activity (Zhang et al., 2009) and anti-apoptotic function (Jiang et al., 2004) in neuronal cells.

However, the effects of catalpol on DOX-induced cardiac damage have not been described. In the present study, we examined for the first time whether catalpol protects cardiomyocytes against DOX–induced cytotoxicity and tried to find out the potential mechanism.

MATERIALS AND METHODS

HUVECs and HeLa cells were obtained from Shanghai Institute of Cell Biology (Shanghai, China). Catalpol was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO BRL Life Technologies (Grand Island, USA). MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corporation (St. Louis, USA). Assay kits for antioxidant enzyme

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activities, MDA test kit and dichlorofluorescein diacetate (DCFH-DA) detection kit were obtained from Beyotime Institute of Biotechnology (Jiangshu, China). LDH-cytotoxic test kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of analytical grade and obtained from Beijing Chemical Factory (Beijing, China).

Cell culture

The H9c2 and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 µg/ml of penicillin and 100 µg/ml of streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Experimental groups

There were five groups in this study: (1) Control group (cells were untreated). (2) DOX group (cells were cultured with the medium containing 4 µmol/L of DOX for 3 h). (3, 4 and 5) Catalpol group (cells were treated with catalpol at the concentration of 0.1, 1 and 10 µg/ml for 24 h and then exposed to 4 µmol/L of DOX for 3 h).

MTT assay

H9c2 cells were incubation with different concentrations of catalpol (0.1, 1, 10, 100 and 1000 µg/ml) for 24 h or 10 µg/ml catalpol for different amounts of time (3, 6, 12, 24 and 48 h) or with different concentrations of DOX (1, 2, 4, 8 and 16 µmol/L) for 3 h or 4 µmol/L DOX for different amounts of time (1, 3, 6, 12 and 24 h). Cell viability was assayed by using a MTT assay. At the end of each treatment period, 20 µl of MTT was added to each well and the microplate was incubated at 37°C for 4 h. The medium with MTT was removed and 150 µl DMSO was added to each well. The absorbance was measured at 490 nm. Wells without cells were used as blanks.

Results were expressed as a percentage of control. HeLa cells were used to investigate the interference of catalpol on the antitumor activity of DOX. HeLa cells were incubation with10 µg/ml catalpol for 24 h or 4 µmol/L DOX for 3 h or pretreatment with10 µg/ml catalpol 24 h then exposed to 4 µmol/L DOX 3 h. Cell viability was assayed by using a MTT assay.

Cytotoxicity assay

The LDH leakage was detected as described by the manufacturers of the LDH-cytotoxic test kit. Briefly, 20 µl of culture medium was incubated with nicotinamide adenine dinucleotide and 2 mM pyruvate at 37°C for 15 min. Then 0.25 ml of 2, 4-Dinitrophenylhydrazone was added and mixed, and then the reaction was stopped by the addition of 2.5 ml of 0.4 M NaOH. The absorbance was read at 440 nm.

Hoechst 33258 staining

Cells were harvested and washed with phosphate buffered saline (PBS) twice and fixed in 4% formaldehyde at 4°C for 10 min. Afterward, the cells were stained with Hoechst 33258 for 5 min in dark at 37°C. Nuclear morphology observed under a fluorescent microscope (Nikon, Yokohama, Japan). Apoptotic cells were identified by condensed, brightly stained nuclei. Cells from five random microscopic fields at 400 magnification were analyzed, and the numbers of apoptotic cells were expressed as percentages of the total cells.

Measurement of ROS and lipid peroxidation

The generation of ROS was determined using DCFH-DA detection kit according to the manufacturer's protocol. Briefly, cells were washed with PBS three times and then incubated with 20 µM DCFH-DA at 37°C for 30 min. The absorbance was read at 525 nm. The MDA was determined by the thiobarbituric acid method using MDA test kit according to the manufacturer's protocol. Briefly, sample was collected and incubated with 200 µl of TBA reagent at 100°C for 15 min. After cooling to room temperature in an ice batch for 5 min, the cells were concentrated by centrifugation at 1000 g for 10 min. 200 µl supernatant was transferred to a 96 well microplate and the absorbance of the colored product was read at 532 nm. Values of MDA level were expressed as nanomoles per milligrams of protein.

Enzymatic activity

The total SOD (T-SOD) activity was determined by the xanthine oxidase method using total superoxide dismutase assay kit according to the manufacturer's protocol. Briefly, sample was collected and incubated with xanthine-xanthine oxidase and nitroblue tetrazolium (NBT) at 37°C for 20 min. The absorbance was read at 550 nm. The SOD activity was expressed as units per milligrams of protein. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

The GPx activity was determined by cellular glutathione peroxidase assay kit according to the manufacturer's protocol. Briefly, sample was collected and incubated with 5 ul of the 10 mM NADPH and 0.4 µl of glutathione reductase. The reaction was initiated by the addition of 4 µl of the 15 mM tert-Butyl hydroperoxide solution. The absorbance was read at 340 nm. The GPx activity was expressed as units per milligram of protein. One unit of GPx activity was defined as the amount required to oxidize 1 µmol NADPH to $NADP^+$ in 1 min at 25°C, pH 8.0.

The CAT activity was determined using CAT assay kit according to the manufacturer's protocol. Briefly, sample was collected and incubated with 10 ul of the 250 mM hydrogen peroxide at 25°C for 5 min. The reaction was terminated by the addition of potassium hydroxide. After incubation with the chromogenic agent at 25°C for 15 min, the absorbance was read at 520 nm. The CAT activity was expressed as units per milligram of protein. One unit of CAT activity was defined as the amount of CAT required to decompose 1 µmol H_2O_2 in 1 min at 25°C, pH 7.0.

Statistical analysis

Data are expressed as mean ± standard deviation (S.D). Statistical comparison within groups was carried out with one way ANOVA, and the Student-Neuman-Keuls test was used to analyze between two groups. Values of $P < 0.05$ are considered statistically significant.

RESULTS

Effect of catalpol and DOX on the survival of H9c2 cells

Treatment with different concentrations of catalpol (0.1, 1 and 10 µg/ml) for 24 h increased the viability of H9c2 cells in a dose-dependent manner. Cell viability was 106.9 ± 3.0%, 114.0 ± 5.6% and 137.0 ± 9.8%, respectively. The protective effect of catalpol was

inhibited and cell viability reduced at a concentration of 1000 µg/ml (Figure 1A). Treatment with 10 µg/ml catalpol for up to 48 h increased the viability of H9c2 cells in a time-dependent manner. Cell viability was 109.9 ± 4.8%, 120.6 ± 4.6% and 128.5 ± 6.5%, 135.4 ± 5.5%, 143.1 ± 6.8%, respectively (Figure 1B). Treatment with different concentrations of DOX (1, 2, 4, 8 and 16 µmol/L) for 3 h resulted in dose-dependent decreases in cell viability. Cell viability was 95.8 ± 3.4%, 82.2 ± 5.5%, 54.9 ± 6.3%, 42.9 ± 4.1% and 28.8 ± 3.2%, respectively (Figure 1C). Treatment with 4 µmol/L DOX for different amounts of time (1, 3, 6, 12 and 24 h) resulted in time-dependent decreases in cell viability. Cell viability was 75.8 ± 3.4%, 54.9 ± 6.3%, 44.8 ± 1.8%, 33.5 ± 3.9% and 25.6 ± 3.4% respectively (Figure 1D). The viability was 54.9 ± 6.3% associated with treatment 4 µmol/L DOX for 3 h and this concentration was used in subsequent experiments.

Catalpol did not interfere with the antitumor activity of DOX

After exposure to 4 µmol/L DOX for 3 h, the viability of HeLa cells significantly reduced compared with the control group (Table 1). Cell viability did not show any significant differences after pretreatment with both catalpol and DOX compared with the DOX group. Moreover, when pretreatment with catalpol alone, cell viability did not decreased significantly compared with control group.

Catalpol increases the viability of H9c2 cells exposed to DOX in a dose-dependent manner

H9c2 cells were pretreated with different concentrations of catalpol (0.1, 1 and 10 µg/ml) for 24 h and then exposed to DOX for 3 h. Cell viability was assayed by MTT (Figure 1E). The survival rate of H9c2 cells was decreased after exposed to 4 µmol/L of DOX for 3 h compared to control group. Catalpol increased the viability of H9c2 cells in a dose-dependent manner at concentration of 0.1 to 10 µg/ml. As shown in Table 3, LDH concentrations in medium from control group cells were minimal. Treatment with 4 µmol/L of DOX for 3 h led to marked increases in LDH levels. Pre-treatment with catalpol (0.1, 1 or 10 µg/ml) attenuated LDH activity significantly in a dose dependent manner.

Catalpol prevents H9c2 cells from DOX–induced apoptosis

Apoptosis was detected using Hoechst 33258 staining. Compared with control group, the apoptotic rate was significantly increased in DOX group. Catalpol (0.1, 1 or 10 µg/ml) significantly reduced the apoptotic rate in a dose dependent manner (Table 2).

Catalpol reduced the levels of ROS and lipid peroxidation

As shown in Table 3, the level of intracellular ROS was measured with fluorescence probe DCFH-DA. Compared with control, the intracellular ROS manifested as fluorescence intensity increased significantly in H9c2 cells exposed to DOX. The fluorescence intensity decreased significantly by pretreatment with catalpol (0.1, 1 or 10 µg/ml) in a dose dependent manner.

As shown in Table 3, the content of MDA increased significantly in the DOX group compared with the control group. Catalpol (0.1, 1 or 10 µg/ml) significantly reduced lipid peroxidation in a dose dependent manner.

Catalpol increased the activity of antioxidant enzymes

Compared with control, notable reductions in CAT, GPx and T-SOD activities were observed after exposure to 4 µmol/L DOX for 3 h. Pretreatment with catalpol (0.1, 1 or 10 µg/ml) significantly elevated the activities of CAT, GPx and T-SOD in a dose dependent manner (Table 4).

DISCUSSION

A lot of traditional plants have been shown to have cardioprotective effects in experimental studies (Shao, 2011; Xing and Jian, 2011; Ritter et al., 2010). Catalpol, listed in the 2010 edition of Pharmacopoeia of the People's Republic of China, is an iridoid glucoside isolated from the root of *R. glutinosa Libosch* and used for the quality evaluation of *R. glutinosa*. Catalpol has many pharmacological actions, such as anti-brain ischemia, anti-senile dementia, promoting neuro-remodeling and reducing capillary permeability (Zhu et al., 2009). However, there have been no studies at all on whether catalpol is able to protect the cardiomyocytes from DOX-induced damage. In this study, we examined the protective effect of catalpol on the H9c2 cells exposed to DOX by MTT assay, LDH release and Hoechst 33258 staining. The major finding of the present study is that pretreatment with catalpol (0.1, 1 and 10 µg/ml) for 24 h markedly reduces the decrease in viability of H9c2 cells exposed to DOX. Catalpol dose-dependently inhibited apoptosis and decreased cytotoxicity in H9c2 cells exposed to DOX. Moreover, catalpol did not interfere with the antitumor activity of DOX in HeLa cells by MTT test. DOX is a widely used antitumour agent; however, its clinical use is limited because of its dose-dependent cardiotoxicity. The production of ROS is considered to be the main

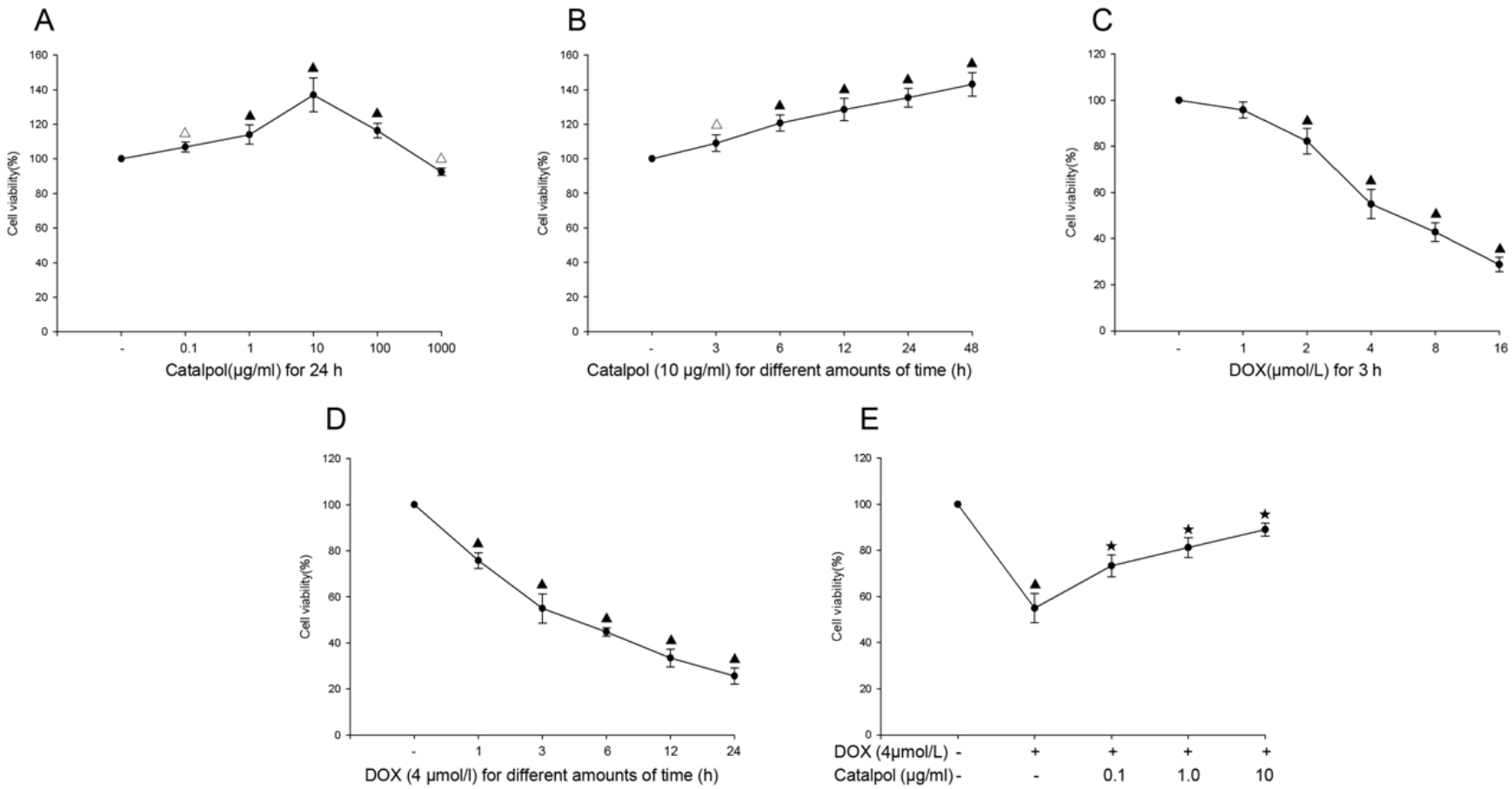

Figure 1. The viability of DOX and catalpol in H9c2 cells. It was measured using MTT assay. (A) The viability in H9c2 cells with different concentrations of catalpol (0.1, 1, 10, 100 and 1000 μg/ml) for 24 h. (B) The viability in H9c2 cells with 10 μg/ml catalpol for different amounts of time (3, 6, 12, 24 and 48 h). (C) Cell viability in H9c2 cells with different concentrations (1, 2, 4, 8 and16 μmol/L) for 3 h. (D) The viability in H9c2 cells with 4 μmol/L DOX for different amounts of time (1, 3, 6, 12 and 24 h). (E) The viability in H9c2 cells with 4 μmol/L DOX for 3 h after pretreatment with different concentrations of catalpol (0.1, 1, 10 μg/ml) for 24 h. The data represent means ± S.D from 5 independent experiments. ⋆P<0.05 and ▲P <0.01 compared to control group. ⋆P <0.01 compared to DOX group.

mechanism of DOX-induced cardiotoxicity (Takemura and Fujiwara, 2007; Li et al., 2011; Fu et al., 2010). ROS can take up electrons from the lipids in cell membranes, resulting in cell damage. This process of oxidation of the fatty membranes is called lipid peroxidation which contributes to oxidant-induced cell death. MDA, a major and stable end product formed of peroxidation, is regarded as marker of lipid peroxidation (Del et al., 2005). Furthermore, ROS can also cause mitochondrial structural and functional damage, which may result in cardiomyocyte apoptosis or

Table 1. HeLa cells were used to investigate the interference of catalpol on the antitumor activity of DOX. Cell viability was assayed by using a MTT assay.

Group	Cell viability (%)
Control	100
Catalpol (10 μg/ml)	97.6 ± 2.4
DOX	29.5 ± 2.9▲
DOX + catalpol (10 μg/ml)	28.6 ± 3.1▲

The data from 5 independent experiments were expressed as means ± S.D. ▲P <0.01 compared to control group.

Table 2. Preventive effects of catalpol against DOX-induced apoptosis in H9c2 cells cell. Apoptotic cells were evaluated by Hoechst 33258 staining. Cells from five random microscopic fields at 400 magnification were analyzed, and the numbers of apoptotic cells were expressed as percentages of the total cells.

Group	Apoptotic rate (%)
Control	93.4 ± 3.9
DOX	58.0 ± 2.7▲
DOX + catalpol (0.1 μg/ml)	63.2 ± 4.0★
DOX + catalpol (1 μg/ml)	71.4 ± 4.5★
DOX + catalpol (10 μg/ml)	80.0 ± 3.2★

The data from 5 independent experiments were expressed as means ± S.D. ▲P <0.01 compared to control group. ★P <0.01 compared to H_2O_2 group.

Table 3. The effects of catalpol on LDH leakage, MDA level, and intracellular ROS under DOX treatment in H9c2 cells. Cells were pretreated with different concentrations of catalpol (0.1, 1 and 10 μg/ml) for 24 h and then exposed to 4 μmol/l DOX for 24 h.

Group	ROS	LDH (U/L)	MDA (nmol/ mg protein)
Control	26.9 ± 3.0	673.6 ± 98.6	1.12 ± 0.20
DOX	119.0±16.8▲	1142.4±128.2▲	3.21± 0.29▲
DOX + catalpol (0.1 μg/ml)	99.0 ± 7.9★	982.0± 71.1★	2.43 ± 0.35★
DOX + catalpol (1 μg/ml)	66.0 ± 10.3★	841.6 ± 91.3★	1.84 ± 0.33★
DOX + catalpol (10 μg/ml)	42.4 ± 6.5★	700.6 ± 87.1★	1.37 ± 0.30★

The data represent means ± S.D. from 5 independent experiments. ▲P <0.01 compared to control group. ★P <0.01 compared to DOX group.

death (Arola et al., 2000; Menna et al., 2007; Kumar et al., 2001; Wu et al., 2002). Thus, the scavenging of excessive ROS by antioxidants may be effective in preventing oxidative cell death. Cells have evolved different antioxidants to neutralize ROS which can suppress lipid peroxidation, hence these antioxidants are absolutely critical for inhibiting oxidative stress-induced cytotoxicity. Antioxidant enzymes, such as CAT, GPx and SOD, are a class of enzymes capable of inhibiting the oxidation and are major intracellular antioxidant defenses in cells. It has been shown that the overexpression of antioxidant enzymes can provide protective effects against the ROS-induced cardiomyocytes damage (Fiers et al., 1999; Liu et al., 2009). In present results, incubation with DOX decreased the activities of CAT, SOD and GPx and increased the concentration of MDA and ROS in H9c2 cells. Catalpol increased the activities of CAT, GPx and SOD, and meanwhile reduced MDA content and scavenged ROS formation. Our findings suggest that catalpol could protect H9c2 cells from

Table 4. The effects of catalpol on the activities of antioxidant enzymes in H9c2 cells.

Group	CAT (U/mg protein)	Gpx (U/mg protein)	T-SOD (U/mg protein)
Control	15.33 ± 2.75	20.14 ± 1.96	8.41 ±1.15
DOX	7.50 ± 1.31▲	13.99 ± 2.30▲	3.85 ± 0.78▲
DOX + catalpol (0.1 μg/ml)	13.43 ± 1.31★	20.62 ± 1.46★	8.29 ± 1.19★
DOX + catalpol (1 μg/ml)	17.97± 1.18★	27.41 ± 1.36★	12.82 ± 2.52★
H_2O + catalpol (10 μg/ml)	23.88 ± 3.06★	34.41 ± 2.84★	19.56 ± 1.87★

All values are means ± S.D. of three replicates. ▲P <0.01 compared to control group. ★P <0.01 compared to DOX group.

DOX-induced cytotoxicity and the underlying mechanisms were due to its antioxidant activity.

In conclusion, our data demonstrates for the first time that catalpol can protect H9c2 cells against DOX-induced cytotoxicity without affecting its antitumor activity. This study suggests that catalpol may serve as an antioxidant for prevention of DOX-induced cardiotoxicity.

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