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(−)-Epigallocatechin-3-gallate alleviates doxorubicin-induced cardiotoxicity in sarcoma 180 tumor-bearing mice

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ABSTRACT

Aims: (−)-Epigallocatechin-3-gallate (EGCG), a major green tea polyphenol compound, plays an important role in the prevention of cardiovascular disease and cancer. The present study aimed to investigate the effects of EGCG on doxorubicin (DOX)-induced cardiotoxicity in Sarcoma 180 (S180) tumor-bearing mice.
Main methods: S180 tumor-bearing mice were established by subcutaneous inoculation of S180 cells attached to the axillary region. The extent of myocardial injury was accessed by the amount of lactate dehydrogenase (LDH) released in serum. Heart tissue was morphologically studied with transmission electron microscopy. Apoptosis, reactive oxygen species (ROS) generation, mitochondrial membrane potential ($\Delta\Psi m$) as well as calcium concentration were measured by flow cytometric analysis. Expression levels of manganese superoxide dismutase (MnSOD) were analyzed by Western blot.
Key findings: Results showed that the combination with EGCG and DOX significantly inhibited tumor growth and enhanced induction of apoptosis compared with DOX alone. Moreover, administration of EGCG could suppress DOX-induced cardiotoxicity as evidenced by alleviating LDH release and apoptosis in cardiomyocyte. EGCG-evoked cardioprotection was in association with the increase of $\Delta\Psi m$ and MnSOD expression. EGCG was also found to attenuate ROS generation and myocardial calcium overload in Sarcoma 180 tumor-bearing mice subjected to DOX.
Significance: EGCG alleviated DOX-induced cardiotoxicity possibly in part mediated by increasing of MnSOD and Ψm, reducing myocardial calcium overload and subsequently attenuating the apoptosis and LDH release. Our findings suggest that co-administration of EGCG and DOX have potential as a feasible strategy to mitigate cardiotoxicity of DOX without compromising its chemotherapeutic value.

1. Introduction

Doxorubicin (DOX: also called adriamycin), anthracycline antibiotic, belongs to the broad spectrum and highly effective anticancer drug. It played a significant role in the treatment of several malignant tumors including leukemia, lymphoma and breast cancer. Although these beneficial anti-tumor effects of DOX are generally recognized, its severe adverse effects and complications still restrict the prognosis of cancer patients by the treatment of DOX [1,2]. It is well established that the most common side effect is cardiotoxicity. It has been demonstrated that a high affinity between DOX and myocardial tissue is the main factor contributing to the DOX-induced severe cardiotoxicity in vivo. DOX-induced cardiotoxicity is in proportion to its administrative dose which often resulted in various cardiac arrhythmias and congestive heart failure. Currently, cumulative and irreversible cardiotoxicity is the major limiting factor of DOX in the clinical setting [3–5].

Oxidative stress resulting from quinone-generated reactive oxygen species (ROS) has been suggested to be a common mechanism involved in DOX-mediated cardiomtopathy. There contains abundant mitochondria in the myocardial tissue, accounting for approximately 40% of the total volume of the heart. Besides, DOX possesses a high affinity for mitochondrial cardiolipin, which resulted in the accumulation of DOX in cardiomyocytes [6,7]. Under the conditions of DOX treatment, the heart becomes a location for redox reaction. Briefly, under the circumstances of NADPH, quinone structure of DOX is transformed into a

Abbreviations: cDCFH-DA, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate; DOX, Doxorubicin; EGCG, (−)-epigallocatechin-3-gallate; LDH, lactate dehydrogenase; $\Delta\Psi m$, mitochondrial membrane potential; MnSOD, manganese superoxide dismutase; Rho 123, Rhodamine 123; ROS, reactive oxygen species; S180, Sarcoma 180.

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seminquinone structure through one-electron reduction in the mitochondrial electron transport chain. Seminquinone structure of DOX could be returned to quinone structure via acting with O_2 to generate a O_2^-. The circulating of DOX between quinone structure and seminquinone structure was in association with the generation of O_2^-, which further led to over-production of ROS. As a consequence, quinone-mediated over-production of ROS contributes to oxidative stress and cardiotoxicity under the conditions of DOX treatment [8–10].

Tea, the most regularly consumed beverage worldwide, received an extensive attention due to its health benefits. (−)-Epigallocatechin-3-gallate (EGCG), identified as a major green tea polyphenol, has been reported to exert a positive effect on cancer chemopreventive activities and cardiovascular support [11]. Chen et al. [12] found that the combination of DOX and EGCG enhanced the DOX-mediated anti-tumor effect in hepatocellular carcinoma cell lines in vitro. In addition, Saeed et al. [13] showed that EGCG preconditioning inhibited myocardial injury caused by DOX in rats, and the mechanism was closely related to its antioxidant activity. EGCG is generally well known for its antioxidant activity, cardioprotection, and anti-tumor effect. However, little is known about whether EGCG could exert protective effects against cardiomyopathy in the tumor-bearing mice associated by the anti-tumor treatment of DOX in vivo.

Owing to the health benefits of EGCG on the anticancer and cardioprotection activities, we hypothesised that it could be regarded as a synergistic agent in combination with DOX to improve the prognosis of cancer patients. Therefore, the present study was aimed to investigate the protective effects of EGCG against DOX-caused cardiotoxicity and further to illuminate the underlying mechanisms in Sarcoma 180 (S180) tumor-bearing mice.

2. Materials and methods

2.1. Animals

Kunming mice [weighing 22.0 ± 2.0 g, Grade II, Certificate Number SCXK (gan) 2006-0001] were purchased from Jiangxi University of Traditional Chinese Medicine, Nanchang, China. Animals were acclimated to our laboratory environment for 1 week. They were housed under standard conditions (temperature 25 ± 2 °C, humidity 50 ± 10%,12 h light, 12 h dark), and were allowed free access to food and water. All animals used in this study were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health, USA (NIH Publication No. 85-23, 1996). All animal procedures were conducted after approval from the Animal Care Review Committee, Nanchang University.

2.2. Cells and cell culture

Murine S180 cell line was purchased from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai. The cells were placed in 60 mm Petri dishes and incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C with 5% CO_2.

2.3. Reagents

DOX was provided by Shenzhen Arcandor's Pharmaceutcal Co., China (Chinese Drug Approval Number: H44024359). (−)-Epigallocatechin-3-gallate was obtained from Sigma Chemical Company (St Louis, MO, USA), and diluted to appropriate stock concentration of 2.5 mg/mL in 0.9% normal saline. RPMI-1640 medium and fetal bovine serum was obtained from Life Technologies (Paisley, Scotland). Fluo-3/AM was obtained from Biotium Company (Hayward, CA, USA). Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA). 5(6)-Carboxy-2′,7′-dichlorofluorescein diacetate (cDCFH-DA) and Rhodamine 123 (Rho 123) were obtained from Molecular Probes Inc. (OR, USA). Annexin V-FITC apoptosis detection kit, lactate dehydrogenase (LDH) kit, Bradford Protein Assay kit, and manganese superoxide dismutase (Mn-SOD) kit were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-Mn-SOD (25 kDa) and anti-β-actin (42 kDa) primary antibodies, as well as the HRP-linked secondary antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The total protein extraction kit was purchased from Beyotime Institute of Biotechnology.

2.4. Experimental groups and protocols

0.2 mL of cell suspension containing one million S180 tumor cells was subcutaneously inoculated into the axillary region. 24 h after subcutaneous inoculation of S180 tumor cells, these mice were described as sarcoma 180 (S180) tumor-bearing mice and was commonly used as a representative animal model [14,15]. Then S180 tumor-bearing mice were randomly divided into 4 groups of eight animals each as follows: control group: S180 tumor-bearing mice injected intraperitoneally (i.p.) once daily with 0.9% sodium chloride (0.2 mL) for 10 consecutive days. DOX group: S180 tumor-bearing mice injected i.p. once daily with 2 mg DOX/kg body weight (0.2 mL) for 10 consecutive days. EGCG group: S180 tumor-bearing mice were injected i.p. once daily with 25 mg EGCG/kg body weight (0.2 mL) for 10 consecutive days. EGCG + DOX group: S180 tumor-bearing mice injected i.p. once daily with 25 mg/kg body weight EGCG (0.1 mL) + 2 mg/kg body weight DOX (0.1 mL) for 10 consecutive days. 24 h after the last EGCG administration, the serum, heart and tumor were collected for the following analyses.

2.5. Analysis for serum LDH

24 h after the last EGCG administration, mice were anesthetized by i.p. with 3.5% chloral hydrate. And then, ophthalmic forceps were used to collected blood samples via the removal of left eyes. 200 μL serum was prepared from blood samples by centrifugation at 2, 000 ×*g* for 5 min. The releases of LDH in serum were measured by an automatic biochemical analyzer (Hitachi 7060, Japan).

2.6. Analysis for cardiac morphology

Mice were killed by cervical vertebra dislocation, and heart was harvested in PBS. Tissues were cut with razor blades from ventricle walls and auricles at sizes appropriate to a transmission electron microscopy (TEM).Then put heart tissue blocks into prechilled glutaraldehyde sodium dimethyl arsenic buffer (pH 7.4) 4 °C for 2 h. Tissues were fixed with Sodium dimethyl arsenic osmium tetroxide buffer. After dehydration in ethanol and acetone in sequential order, specimens were infiltrated with epoxy resin 812, and ultrathin sections were obtained with a ultramicrotome. Finally, heart tissues were double stained with Uranyl acetate and Lead citrate and stained sections were visualized and imaged under a Hitachi-800 TEM (Scotia, NY).

2.7. Analysis for MnSOD

Standard procedure was followed for the preparation of 10% myocardial tissue homogenate and the supernatants were collected. The Bradford protein assay kit was used to determine the protein contents in the supernatants. The activities of SOD were then measured following their detection kit instructions.

2.8. Analysis for apoptosis

Cardiomyocytes were prepared from the myocardial tissues of S180-bearing mice according to Reinecke et al. [16]. Meanwhile, the murine S180 cells were isolated from the tumor tissues of S180-bearing mice [17]. Annexin V-FITC apoptosis detection kit was used to determine

apoptosis by staining with Annexin V-FITC and PI. Cardiomyocytes and murine S180 cells were collected and adjusted to a final concentration of approximately 2×10^6 cells/mL. Lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L EDTA, 0.5% Triton X-100] supplemented with 5 μL Annexin V-FITC and 5 μL PI was applied to incubate and stain the cells. Stained cells were analyzed by flow cytometry with a FACS ort cell sorter (Becton Dickinson, San Jose, CA, USA).

2.9. Assays for mitochondrial membrane potential ($\Delta\Psi m$)

In the $\Delta\Psi m$ assay, cardiomyocytes (2×10^6 cells/mL) were harvested and suspended in PBS supplemented with 5 μL Rho 123 (1:1000) at 37 °C for 30 min. The changes of $\Delta\Psi m$ were measured by Rho-123 fluorescence using flow cytometry analysis.

2.10. Assays for ROS generation

Cardiomyocytes (2×10^6 cells/mL) were suspended in PBS and stained with 10 μM DCFH-DA at 37 °C for 20 min. The changes of ROS generation were determined by DCF fluorescence using flow cytometry analysis.

2.11. Measurement of calcium concentration

The levels of calcium concentration in cardiomyocyte were determined by staining with a calcium-sensitive dye, fluo-3/AM (Molecular Probes). Cardiomyocytes (2×10^6 cells/mL) were suspended in tyrode solution (135 mM NaCl, 4 mM KCl, 1 mM MgCl, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES. (pH 7.3) supplemented with 5 μL 20 μM membrane-permeant acetoxymethyl ester of the dye at 37 °C for 50 min. Stained cells were analyzed by flow cytometry analysis.

2.12. Protein preparation and Western blotting

The heart tissues were harvested and used for the isolation of proteins by the total protein extraction kit. Western blotting was further used to analyse protein expression. Standard procedure was followed for the separation of proteins using SDS-PAGE. The separated proteins were then electroblotted onto PVDF membranes. The membranes were blocked with nonfat milk, incubated with primary antibodies (Anti-Mn-SOD and anti-β-actin) and secondary antibodies. Blots were assayed using ECL kit and analyzed by the scan densitometric analysis.

2.13. Statistical analyses

Data are expressed as means ± SEM. One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was used to determine the differences in both variables. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of EGCG on DOX-mediated anti-tumor effect in S180 tumor-bearing mice

As shown in Fig. 1A, S180 tumor-bearing mice were administrated with DOX alone or the mixture of DOX and EGCG, the tumor growth was found to be reduced. The combined regime was superior to DOX treatment alone. Additionally, we used flow cytometry analysis to quantify the apoptosis in murine S180 cells derived-tumor tissues. There was a significant increase of apoptosis in DOX group when compared with control group (Fig. 1B and C). As compared with the DOX group, the combination of EGCG + DOX resulted in a remarkable enhancement of apoptosis in murine S180 cells. These findings suggested that EGCG excreted a synergistic effect on the DOX-triggered anti-tumor effect by elevating the tumor cell apoptosis in S180 tumor-bearing mice.

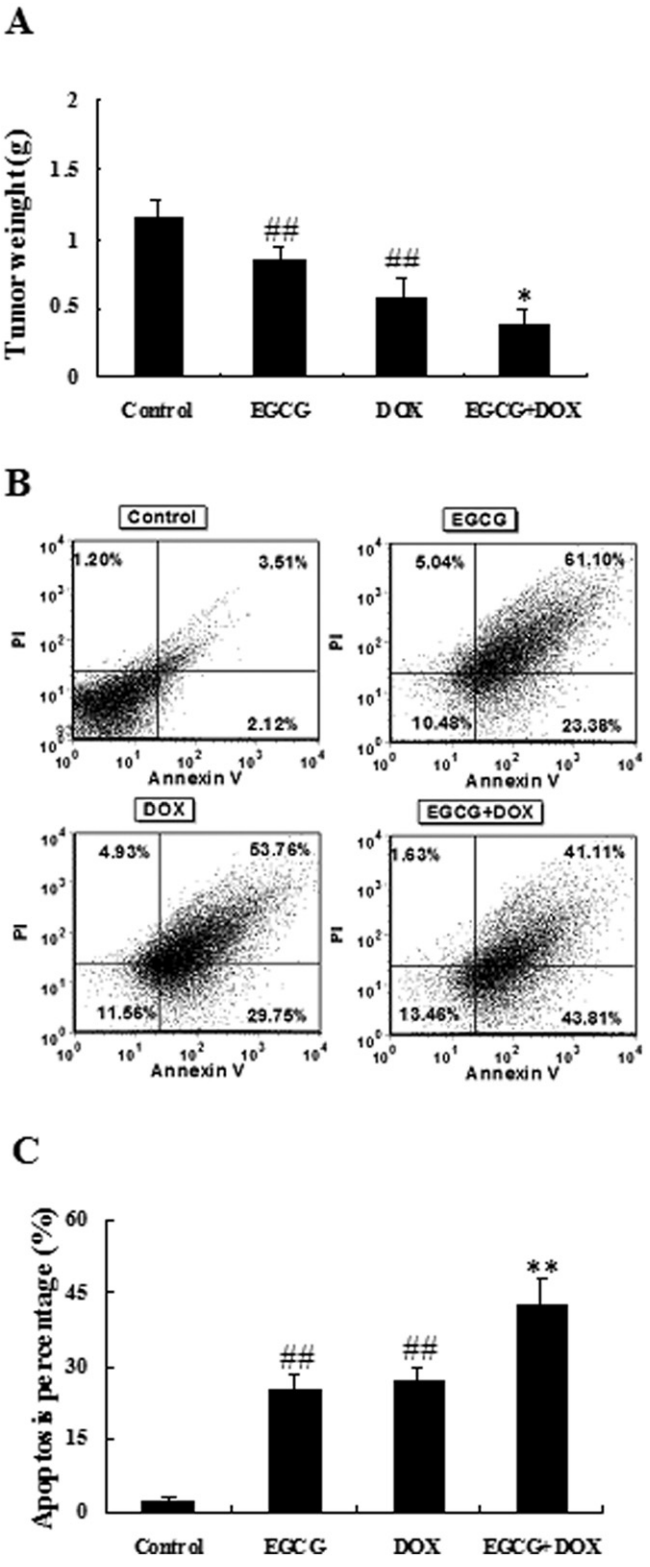

Fig. 1. Effect of EGCG on DOX-mediated anti-tumor activity in S180 tumor-bearing mice. (A) The levels of tumor weight in S-180 tumor-bearing mice by the treatment with EGCG or DOX. (B) and (C) Flow cytometric analysis of apoptosis in the tumor tissue of S180 bearing mice. (B) Detection of apoptotic cells by Annexin V-FITC and PI double staining. (C) Column bar graph of apoptosis. EGCG synergistically affected DOX-inhibited tumor growth by activating tumor cell apoptosis in S180 tumor-bearing mice. $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$. $^{**}P < 0.01$ compared to CTX group. $^{\#\#}P < 0.01$ vs control group.

3.2. Effect of EGCG on DOX-induced morphological alterations of the heart tissue

TEM analysis revealed the morphological alterations of the heart tissue. As shown in Fig. 2, the structure of the bundles of cardiomyocytes was intact regular and neat in control group. Additionally, abundant mitochondria with undisturbed mitochondrial crest and uniform distributed nuclear chromatin were also presented. EGCG group showed an analogical structure referring to the morphology of cardiomyocytes and mitochondria. However, DOX group was found to have distinct electron microscopic myocardial alterations, including swollen mitochondria, mitochondrial crest disruption, and loss of mitochondrial membrane integrity. Compared with DOX group, EGCG + DOX group showed slight damage on the structure of cardiomyocytes. Besides, the arrangement of mitochondria and the integrity of mitochondrial crest showed a slight swelling and vacuolization.

3.3. Effect of EGCG on DOX-mediated cardiotoxicity markers in S180 tumor-bearing mice

Activities of LDH were significantly enhanced in the serum of DOX group, as compared to control group. There were no significant changes of LDH in EGCG group when compared to control group. As compared to DOX group, the release of LDH decreased significantly in EGCG + DOX group (Fig. 3A).

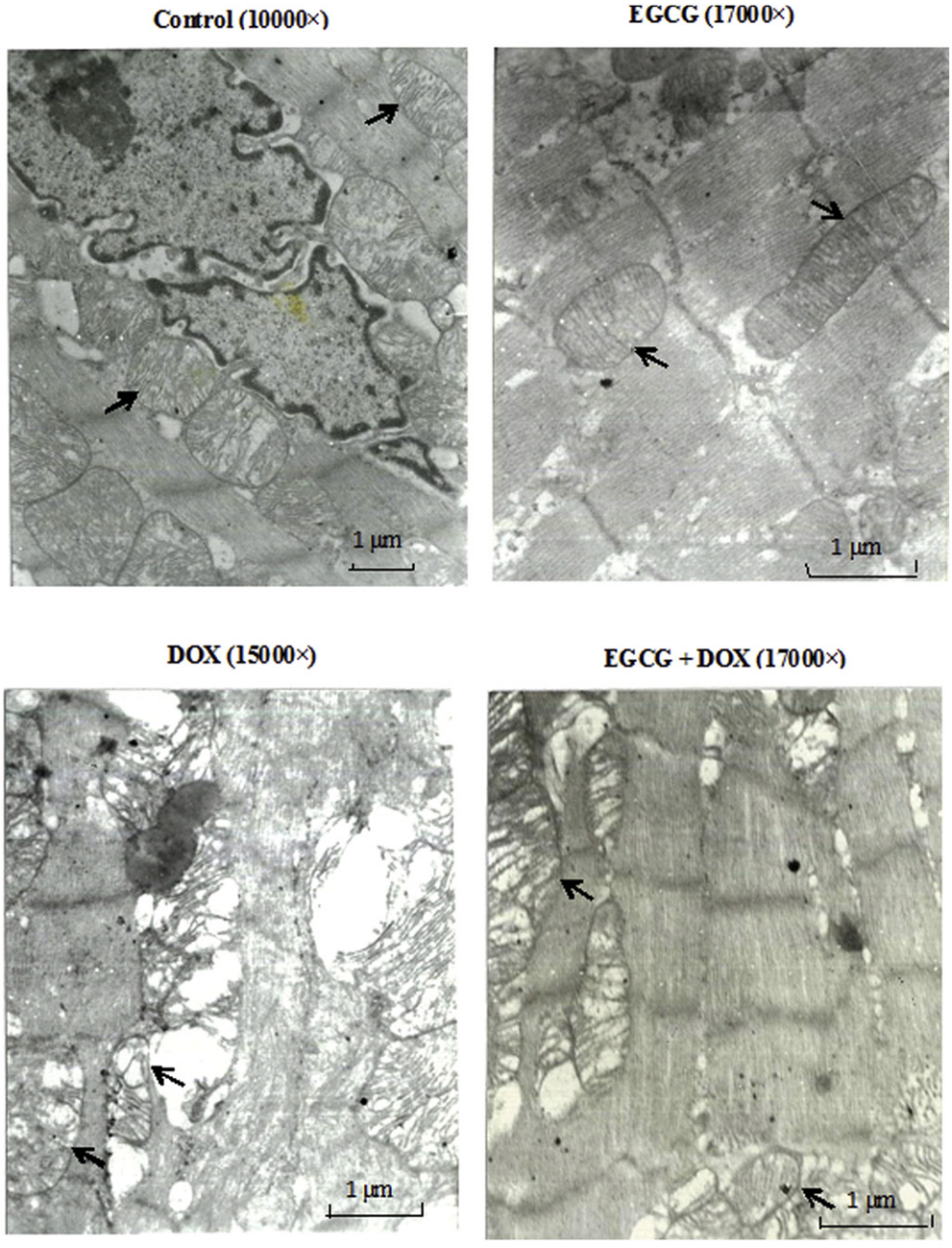

Fig. 2. Effect of EGCG on DOX-induced morphological alterations of the heart tissue. TEM images of cardiomyocyte depicting control group, EGCG group, DOX group, EGCG + DOX group. Control group and EGCG group show the normal myofilament bundles and intact mitochondria with clearly visible mitochondrial crest. Moreover, the arrangement of chromatin was well-distributed. In DOX group, there was no manifestation of intact myocardial fibers bundles while swollen mitochondria and distributed mitochondrial crest were notable. Compared with DOX group, EGCG + DOX group showed regular myofilament bundles and mild mitochondrial edema.

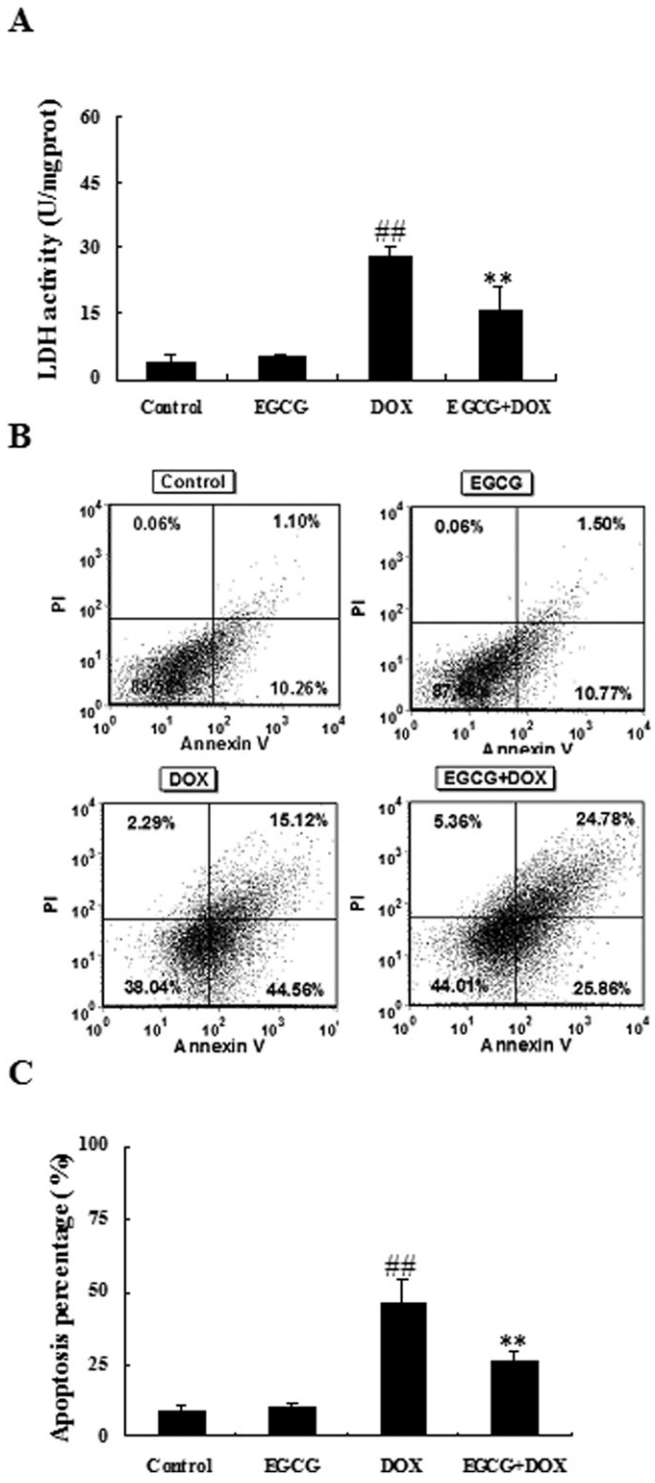

Fig. 3. Effect of EGCG on LDH release and apoptosis in S180 tumor-bearing mice subjected to DOX. (A) Effects of EGCG on the release of LDH in the serum of S180 tumor-bearing mice. (B) and (C) Flow cytometric analysis of apoptosis in the cardiomyocytes isolated from myocardial tissue of S 180 tumor-bearing mice. (B) Detection of apoptotic cells by Annexin V-FITC and PI double staining. (C) Column bar graph of apoptosis. EGCG greatly inhibited excessive LDH release and cardiomyocyte apoptosis in S 180 tumor-bearing mice subjected to DOX. $^{\#\#}P < 0.01$ vs control group, $^{**}P < 0.01$ compared to CTX group.

As shown in Fig. 3B and C, compared to control group, a remarkable elevation of apoptosis was found in DOX group. Administration of the mixture of EGCG + DOX was found to inhibit the activation of apoptosis triggered by DOX. Meanwhile, the levels of apoptosis were not significantly different between EGCG group and control group.

3.4. Effect of EGCG on DOX-mediated oxidative stress markers in S180 tumor-bearing mice

As shown in Fig. 4, ROS generation was significantly increased in DOX group when compared to control group. Interestingly, a mixture of EGCG + DOX induced a remarkable attenuation in oxidative stress, as evidenced by the decreases in ROS generation in the heart tissue. Meanwhile, S180 tumor-bearing mice by the administration of alone, there was not significant difference in ROS levels between EGCG alone group and control group.

3.5. Effect of EGCG on DOX-mediated mitochondrial dysfunction in S180 tumor-bearing mice

As shown in Fig. 5, compared with control group, Rho 123 fluorescence was decreased remarkably in DOX group. Rho123 fluorescence was significantly increased in EGCG + DOX group. Treatment of EGCG alone did not evoke any change in the level of $\Delta\Psi m$ in the tumor-bearing mice when compared to control group.

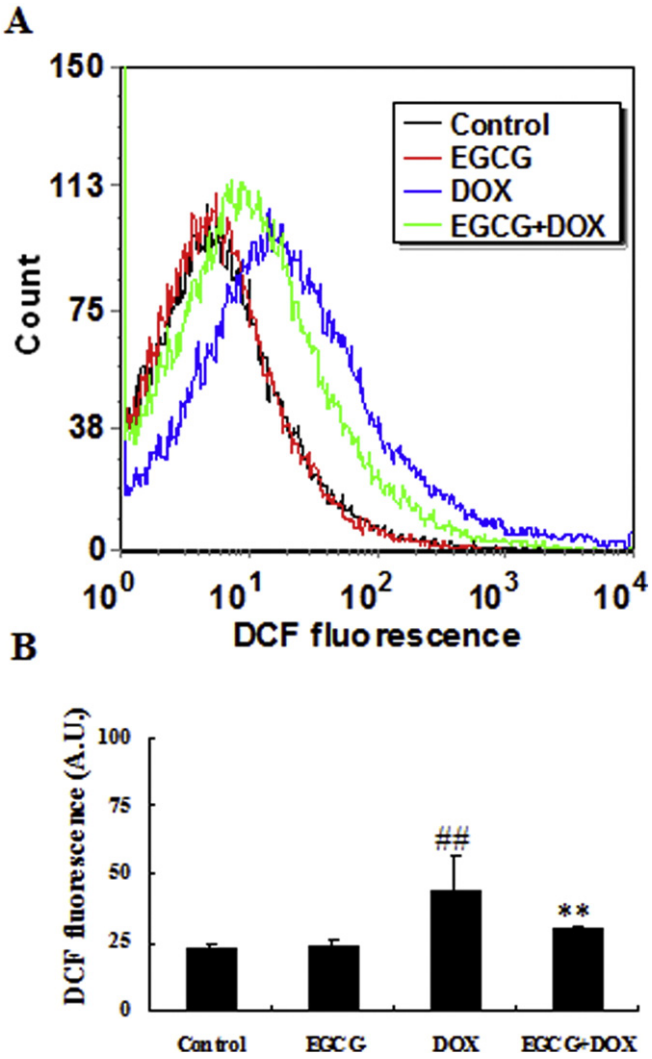

Fig. 4. Effect of EGCG on DOX-induced oxidative stress in the heart tissue. Effects of EGCG on the ROS production in the cardiomyocytes of S180 tumor-bearing mice subjected to DOX. ROS generation was evaluated by the fluorescence of DCF. (A) Flow cytometric graphs of DCF fluorescence; (B) The histogram of DCF fluorescence. $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ vs DOX group.

3.6. Effect of EGCG on myocardial MnSOD in S180 tumor-bearing mice

As shown in Fig. 6, DOX group exhibited a significant decrease in the expression of MnSOD compared with control group. Treatment with EGCG + DOX significantly up-regulated the expression of MnSOD compared with DOX group. Also the mice treated with EGCG did not display any obvious change in the expression of MnSOD when compared to control group.

3.7. Effect of EGCG on myocardial calcium in S180 tumor-bearing mice

The concentration of calcium in DOX group was significantly elevated in cardiomyocytes when compared to control group (Fig. 7). Interestingly, the concentration of calcium in cardiomyocytes from EGCG + DOX group was significantly lower than that from DOX group. Meanwhile, there was not significant difference in the concentrations of calcium between control group and EGCG group.

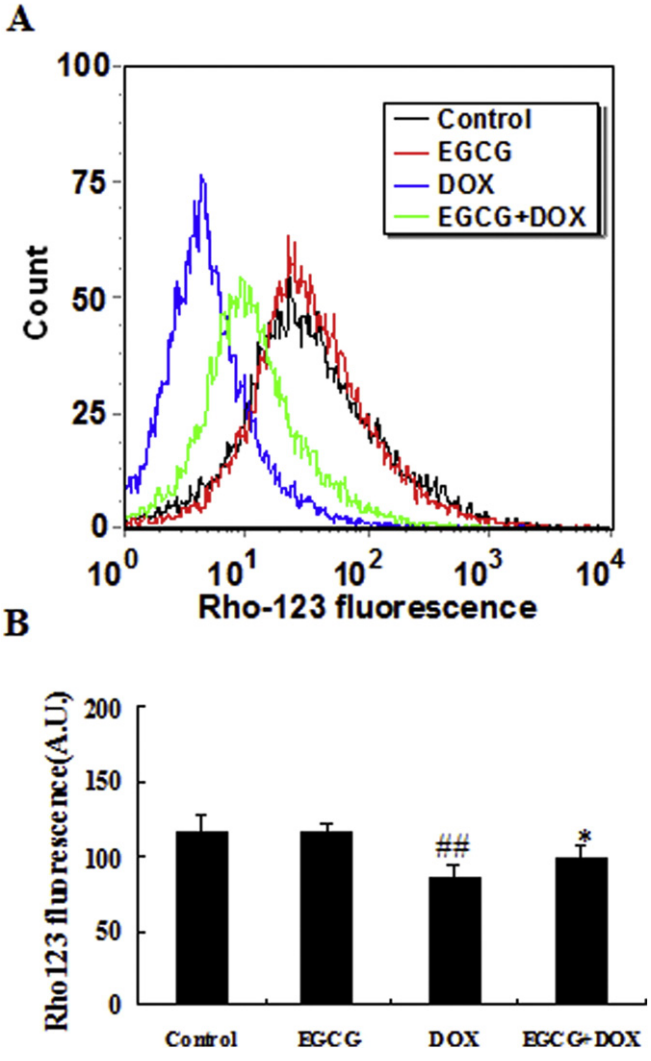

Fig. 5. Effect of EGCG on DOX-induced the loss of mitochondrial membrane potential (ΔΨm) in cardiomyocytes. Flow cytometric analysis was used to determine the level of ΔΨm. (A) Flow cytometric graphs of Rho 123 fluorescence in cardiomyocytes; (B) The histogram of the fluorescence for Rho 123. $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$ vs DOX group.

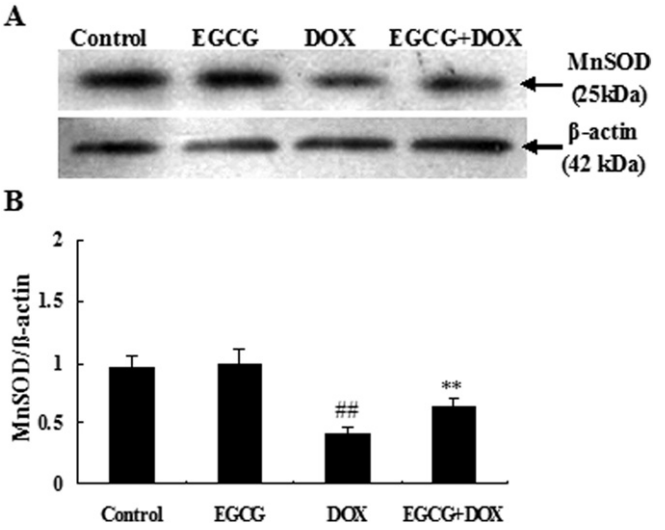

Fig. 6. Effect of EGCG on MnSOD expression in the heart tissue of S180 tumor-bearing mice subjected to DOX. (A) Heart tissues were lysed and protein expression levels of MnSOD and β-actin were analyzed by Western blot. (B) Blots were scanned and expression levels of MnSOD and β-actin were quantified by densitometric analysis. The ratios for these proteins are shown. $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ vs DOX group.

4. Discussion

Collateral injury, such as severe adverse effects and complications in conventional cancer chemotherapy generally caused the paradox referring to the cancer treatment by restricting therapeutic dosages and by impairing the outcome of patients. Cardiotoxicity is a representative complication among the DOX-induced collateral damages in noncancerous tissues [6,18,19]. Novel therapeutic strategies with new concepts are urgently desired to attenuate the cardiotoxicity of DOX without compromising its anti-cancer activity. In the present study, we have demonstrated for the first time that EGCG alleviated DOX-induced cardiotoxicity was associated with the synergistic effects in combination with DOX against tumor growth.

Cardiotoxicity is known for the major problem of DOX in the clinical setting due to its complex action on the cardiovascular system. Although the molecular mechanisms responsible for DOX-induced cardiotoxicity are enigmatic, oxidative stress has been largely implicated in the pathogenesis of DOX-mediated cardiotoxicity [20]. In the heart, there are multiple processes contributing to DOX-induced generation of ROS. Briefly, during the myocardial metabolism of DOX, DOX aglycone and doxorubicinol (DOXol) were generated by the cleavage of the sugar residue and the reduction of the cardonyl family at C13, respectively. The redox cycling of DOX from quinine to seminquinone and DOX metabolites gave rise to ROS via producing O_2^{-1} and H_2O_2. Simultaneously, ROS and DOXol interfered with the normal iron metabolism, which subsequently also resulted in ROS overproduction [21,22]. In this work, DOX significantly increased ROS generation, LDH activity and apoptosis. These results suggested that oxidative stress resulting from the over-production of ROS plays an important role in the pathogenesis of cardiomyopathy caused by DOX. In addition to oxidative stress, cellular senescence was also proven to be another vital contributor to DOX-caused cardiovascular diseases [23–25]. The mechanism of DOX-induced cellular senescence and how to prevent DOX-induced cellular senescence still need more researches.

EGCG, a major constituent of green tea extract, is widely used for the reduction of the incidence of cancer and cancer treatment. Previous studies have demonstrated that EGCG induced apoptosis, subsequently inducing cell death in many carcinoma cells both in vitro and in vivo [26]. Additionally, EGCG possesses strong antioxidant and cardiovascular support activities. Several investigations indicated that the beneficial cardiovascular effects of EGCG are linked to its antioxidant activity [27].

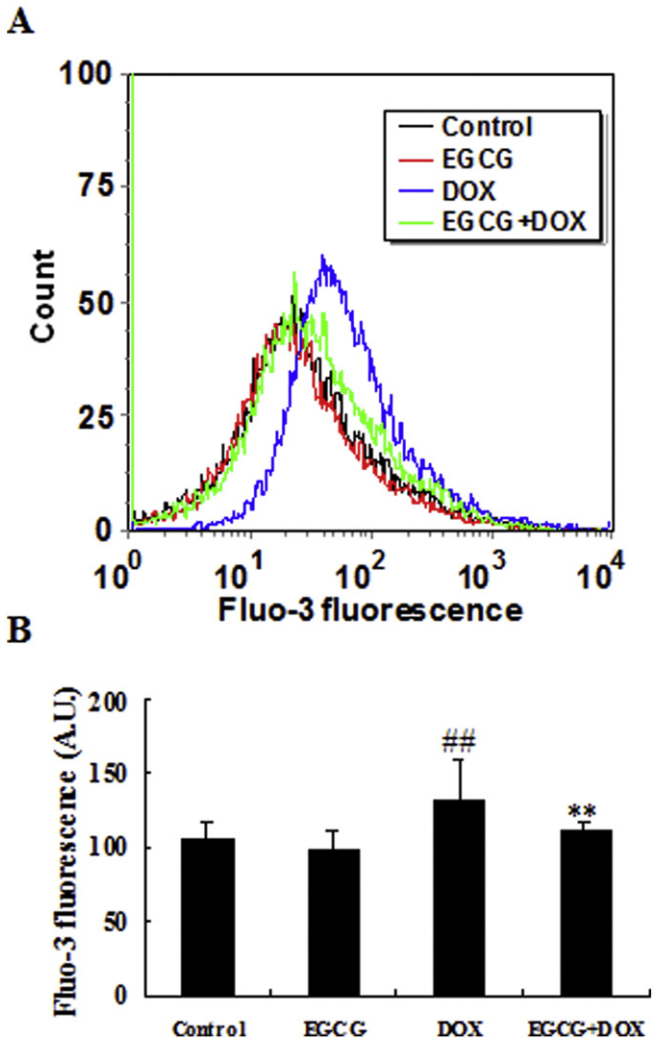

Fig. 7. Effect of EGCG on myocardial calcium in S180 tumor bearing mice subjected to DOX. Concentration of myocardial calcium was determined by flow cytometric analysis with calcium-sensitive dye fluo-3/AM labeling. The concentration of myocardial calcium was in parallel with fluo-3 fluorescence. (A) Flow cytometric graphs of fluo-3 fluorescence in cardiomyocytes; (B) The histogram of the fluorescence for fluo-3 fluorescence. $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs EGCG + DOX group.

The current study aimed to evaluate the synergistic and cardioprotective effects of EGCG in combination with DOX in vivo using S180 tumor-bearing mice. Our results showed that the inhibition effect of EGCG on tumor growth was associated with the increased apoptosis in S180 cells, and further confirmed that EGCG exerted a synergistic effect on the DOX-mediated anti-tumor effect. In the heart, EGCG alleviated DOX-induced cardiotoxicity via the reduction of LDH release and apoptosis in cardiomyocytes. Moreover, our data showed that the protective effects of EGCG against DOX-induced induced cardiotoxicity were in association with the reduction of ROS generation. These findings indicated that EGCG-evoked attenuation of oxidative stress may contribute to its cardioprotective effects.

It was reported that oxidative stress induced calcium leakage and activated calcium-dependent protease [28], and DOX-mediated cardiotoxicity was often associated with the elevation of intracellular calcium level. Disequilibrium of calcium homeostasis induced by DOX played a crucial role in cardiotoxicity via the apoptotic pathway activation [29]. In the present study, a significant increase of calcium level was found in DOX group, Interestingly, EGCG dramatically inhibited DOX-evoked calcium overload, indicating that the control of calcium level may contribute to cardioprotective effects of EGCG against DOX-induced cardiotoxicity in S180 tumor-bearing mice.

Mitochondria play a vital role in calcium regulation and apoptosis involved in DOX-induced cardiotoxicity. In the heart, cardiomyocytes are rich in mitochondria, which account for approximately 40% of the total intracellular volume. DOX has a high affinity for mitochondria via the incorporation of cardiolipin, resulting in the accumulation of DOX in cardiomyocytes [30]. On the other hand, mitochondria are the locus of basal ROS generation through the mitochondrial respiratory chain, and thus subjected to a relatively high oxidative damage when compared to other subcellular organelles [31]. It is generally believed that cardiac mitochondria are the main target of toxicity during DOX chemotherapy [32]. Therefore, to address the role of EGCG in the cardiac mitochondria subjected to DOX, we then analyzed the $\triangle\Psi m$ which plays an essential role in maintaining the mitochondrial function including ATP generation and electron transport. In this study, the administration of DOX was found to evoke a low Rho 123 staining in cardiomyocytes, suggesting the collapse of $\triangle\Psi m$. Meanwhile, EGCG strongly inhibited DOX-induced mitochondrial dysfunction in the heart of S180 tumor-bearing mice. Mitochondrial antioxidant enzymes were beneficial for the survival of aerobic life through scavenging ROS. MnSOD, a primary antioxidant enzyme in mitochondria, is essential for the stability of mitochondria [33,34]. It was found that the expression level of MnSOD in the heart from EGCG + DOX group was higher than that from DOX group. Furthermore, treatment of S180 tumor-bearing mice with EGCG + DOX significantly inhibited DOX-mediated cardiotoxicity. These findings suggested that EGCG could exert its cardioprotective effects through inhibition of mitochondrial damage by increasing the expression of MnSOD.

5. Conclusion

In conclusion, it has been demonstrated for the first time that EGCG enhanced the anti-tumor activity of DOX and ameliorated DOX-induced myocardial oxidative stress in S180 tumor-bearing mice. EGCG-exerted heart benefits were related to the reduction of LDH release, apoptosis, ROS generation and calcium level, and the increase in $\triangle\Psi m$ and expression of MnSOD (Fig. 8). Taken together, our findings supported that co-administration of EGCG and DOX could be considered as a feasible strategy to reduce cardiotoxicity of DOX without compromising its chemotherapeutic value.

Author contributions

"Ming-Yong Xie" contributed to conception and design of the study, generation and approval of the final version of the manuscript. "Yu-Fei Yao" contributed to generation, collection, assembly, analysis and/or interpretation of data, and drafting and revision of the manuscript. "Xiang Liu, Wen-Juan Li, Zi-Wei Shi, Yu-Xin Yan, Le-Feng Wang, and Ming Chen" contributed to generation, collection, assembly, analysis and/or interpretation of data.

Conflict of interest

All authors declare that there are no conflicts of interest.

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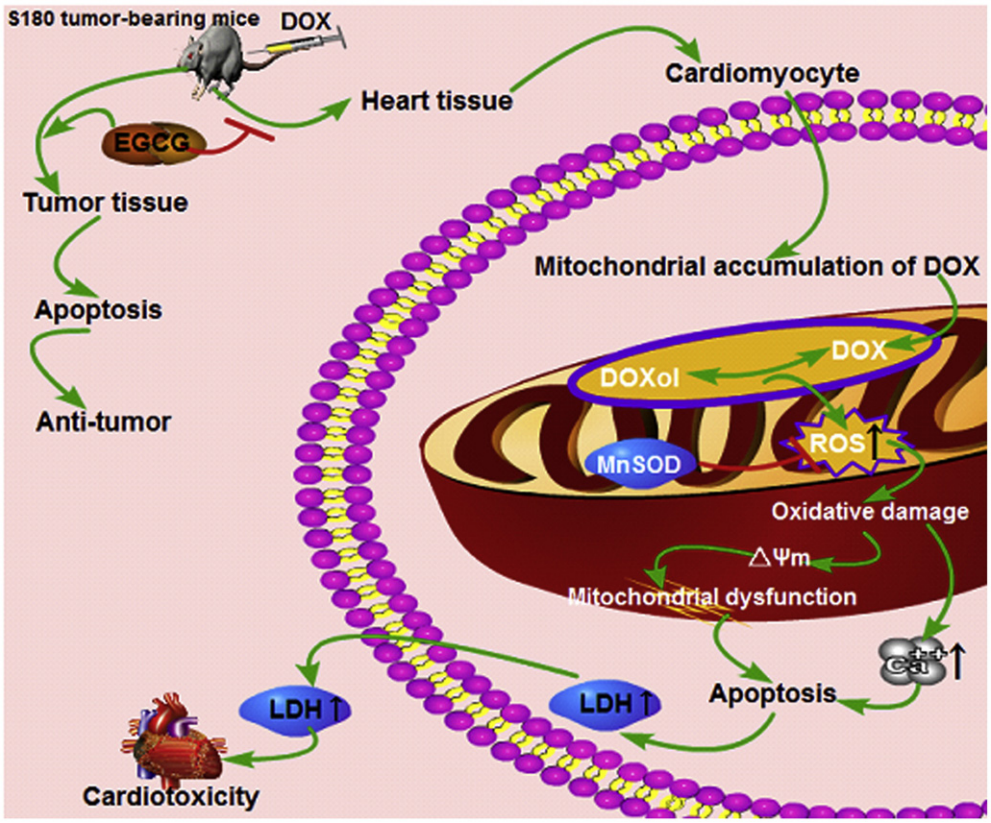

Fig. 8. Proposed model for EGCG-induced cardiovascular support and anti-tumor activities. The schematic model showed that EGCG triggered a synergistic effect on DOX-mediated anti-tumor activity. On the other hand, EGCG could suppress DOX-evoked cardiotoxicity in S180 tumor-bearing mice. In short, DOX had a high affinity for mitochondria, leading to the mitochondrial accumulation of DOX in cardiomyocytes. In the mitochondria, the excessive ROS were generated from the redox cycling of DOX and its metabolites (DOXol). Accordingly, oxidative damage resulting from the redox cycling of DOX was blocked by EGCG via the increase of MnSOD expression. Moreover, EGCG also significantly suppressed the myocardial calcium overload and the loss of △Ψm, and subsequently alleviated the cardiomyocyte apoptosis and LDH release.

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