

Protective effects of psoralen polymer lipid nanoparticles on doxorubicin - induced myocardial toxicity

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Doxorubicin (DOX) induced myocardial toxicity may limit its therapeutic use in clinic. Psoralen (PSO), a major active tricyclic furocoumarin extracted from *Psoralea corylifolia*, is widely used as an antineoplastic agent in treatment of leukemia and other cancers. This study is aim to find the protective effect of psoralen polymer lipid nanoparticles (PSO-PLN) on doxorubicin-induced myocardial toxicity in mice. The model of myocardial toxicity induced by DOX was established. The experiment was divided into 6 groups: normal saline group, DOX + Sulfotanshinone Sodium, DOX + PSO-PLN (3 mg/kg), DOX + PSO-PLN (6 mg/kg), DOX + PSO-PLN (9 mg/kg), DOX group. DOX alone treated mice lead to a significant decrease in the body weight, heart weight, and increase in the serum levels of lactate dehydrogenase (LDH), creatine kinase (CK) and malondialdehyde (MDA) markers of cardiotoxicity. However, DOX reduced glutathione (GSH) content and activities of antioxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxidase (GPX), were recovered by PSO-PLN. And PSO-PLN also decreased markers of cardiotoxicity in the serum. Western blotting data showed that the protective effects of PSO-PLN might be mediated via regulation of protein kinase A (PKA) and p38. Our study suggest that PSO-PLN possesses antioxidant activities, inactivating PKA and p38 effect, which in turn protect the heart from the DOX-induced cardiotoxicity.

Keywords: Doxorubicin. Cardiotoxicity. Psoralen. Psoralen polymer lipid nanoparticles. Antioxidant.

INTRODUCTION

Doxorubicin (DOX) is a effective and potent broad-spectrum anthracycline antibiotics, widely used for the treatment of wide range of cancers including hematological malignancies, carcinomas, and sarcomas (Jain *et al.*, 2011). Unfortunately, Doxorubicin is associated with dose-dependent acute or chronic cardiotoxicity, which

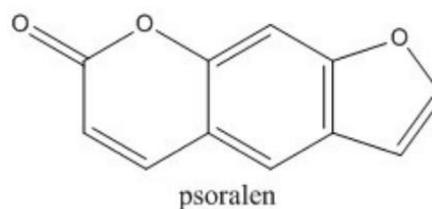
is characterized by hypotension, tachycardia, arrhythmia, transient depression of left ventricular function, and even refractory late-onset cardiomyopathy (Cao *et al.*, 2014). Because of such negative effects, the clinical usage of Doxorubicin is limited despite its potent and effective functions in treating cancer (Abushouk *et al.*, 2017; Cao *et al.*, 2016). Many mechanisms of doxorubicin cardiotoxicity have been elucidated, including triggering reactive oxygen species (ROS) production, mediating intracellular iron accumulation, inducing cell death, activating innate immune system, altering cardiac-specific gene expression and interfering with the cardiac stress response, inducing ubiquitin-proteasome system (UPS) activity, inhibiting neuregulin/ErbB signaling and inhibiting cardiac cell renewal and vasculogenesis (Tacar, Dass, 2013; Wang

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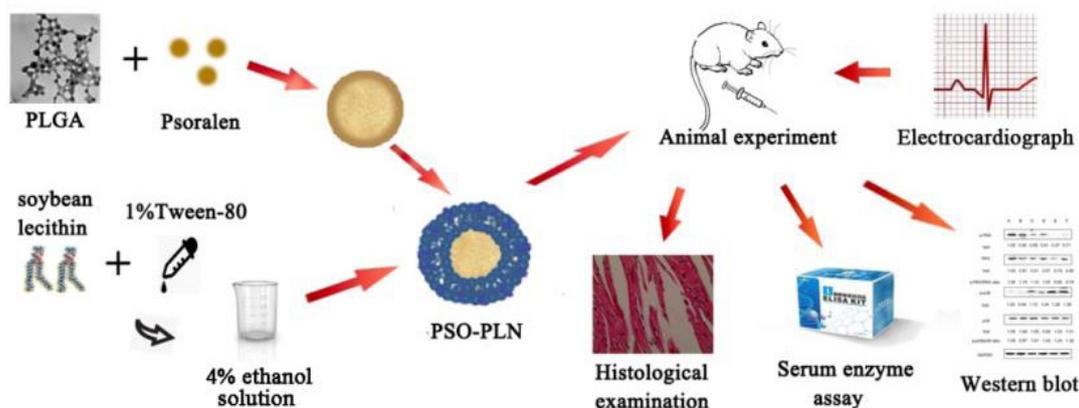
et al., 2014; Yu *et al.*, 2018; Shi *et al.*, 2011; Zhang *et al.*, 2012). Although multiple mechanisms involved in doxorubicin cardiotoxicity have been studied, no single drug has hitherto been able to completely prevent doxorubicin cardiotoxicity in clinical.

Psoralen (PSO), a major active tricyclic furocoumarin extracted from *Psoralea corylifolia*, is widely used as an antineoplastic agent in treatment of leukemia and other cancers (Jiang, Xiong, 2014; Wu *et al.*, 2013; Wang *et al.*, 2016). In previous experiments, we found that psoralen can improve cardiac toxicity caused by doxorubicin in nude mice. But PSO has poor water solubility and low bioavailability. Thus, we investigated

the potential benefit of using PSO in polymeric lipid nanoparticle formulations to improve DOX-induced cardiotoxicity (Huang *et al.*, 2018).



Structural formula of PSO



Graphical abstracts

MATERIAL AND METHODS

Material

Doxorubicin hydrochloride (99.9%) was obtained from Dalian Meilun Biotech Co., Ltd. (China). PSO (98%) was purchased from Nanjing Spring and Autumn Biological Engineering Co., Ltd. (China). PLGA (50:50) was purchased from Jinan Daigang Biomaterial Co., Ltd. (China). Soybean lecithin (injection grade) was obtained from Tai Wei Shanghai Co., Ltd. (China). Tween-80 was purchased from Aladdin (China). Sulfotanshinone Sodium Injection was provided by Shanghai No.1 Biochemical & Pharmaceutical Co., Ltd. (China). Pentobarbital Sodium was purchased from Shanghai

Univ Biotech Co., Ltd. (China). Lactate dehydrogenase assay kit, Creatine kinase assay kit, Troponin Assay Kit, Malondialdehyde (MDA) assay kit, Superoxide Dismutase (SOD) assay kit (WST-1 method) and Reduced glutathione (GSH) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). GAPDH (14C10) Rabbit mAb, PKA C- α (D38C6) Rabbit mAb, Phospho-PKA Substrate (100G7E) Rabbit mAb, p38 MAPK (D13E1) XP[®] Rabbit mAb, Phospho-p38 MAPK (D3F9) XP[®] Rabbit mAb, and all these rabbit anti-mouse monoclonal antibody were purchased from Cell Signaling (USA). HRP-labeled goat anti-rabbit G secondary antibody was obtained from Abcam (USA). Ethanol and acetone as analytical purity, all other reagents were of chromatographic grade.

Animals

36 female Balb/c nude mice (16-20 g, 4–5 weeks old) were provided by Jinan Pengyue Experimental Animal Breeding Co., Ltd., license number: SCXK (Lu) 20140007. All nude mice were housed under standard conditions (room temperature 23±2 °C, humidity 60±15%, 12 h/12 h light/dark cycles) and given free access to standard rodent chow and water. All experimental procedures were performed in accordance with the Guidelines for Animal Experiments from the Committee of Medical Ethics, National Health Department of China.

Preparation of PSO Loaded PLN

PSO-PLN was prepared using the emulsification evaporation-low temperature solidification method (Huang *et al.*, 2018). Both (15 mg) PLGA and (3 mg) PSO were dissolved in an organic solvent (acetone) to form an organic phase. A total of soy lecithin (50 mg) and 1% Tween-80 were dissolved in 4% ethanol solution to form the water phase and heated to 75 °C. Subsequently, the organic phase was slowly injected into the aqueous phase and stirred for approximately 1 h at 75 °C. The concentrated emulsion was added to 15 mL of ice water and stirred at 20 rpm for 1 h. The obtained PSO-PLNs were centrifuged at 1000 rpm for 5 min. The supernatant was filtered through a 0.45 µm membrane filter and stored at 4 °C.

Animal experiments

The animals were acclimatized for one week before experiments. Then 36 nude mice were randomly divided into six treatment groups: sham (saline n=6), DOX (3 mg/kg n=6), DOX+ Sulfotanshinone Sodium (SS, 3 mg/kg n=6), DOX+PSO-PLN (3 mg/kg n=6), DOX+PSO-PLN (6 mg/kg n=6), DOX+PSO-PLN (9 mg/kg n=6). All animals were weighed before the experiment and after the 24 days treatment period. DOX was given every 7 days for a total of 3 times, meanwhile the medicine was given every 3 days for a total of 7 times. The saline group was kept as the control without any treatment. The DOX group received free DOX (3 mg/kg) intravenous injection once every 7 days. The DOX+ SS group received free DOX

(3 mg/kg) intravenous injection once every 7 days, and simultaneously injected intravenously with SS (3 mg/kg) every 3 days. The DOX+PSO-PLN group were treated DOX in stated doses and with the combination of PSO-PLN at different dosages of 3, 6, 9 mg/kg i.v. every 3 days.

Electrocardiography Recording

Electrocardiography(ECG) was recorded at the beginning of the experiment to ensure the normal ECG pattern of the mice. At the end of the 24 days treatment, ECG test was taken 1 hour after the mice were given the medicine by Bioscience ECG recorder (Bioscience, Washington, USA). Anesthesia was assessed clinically by pedal reflex. And anesthetized mice were placed in the supine position on a board. Then, needle electrodes were inserted beneath the skin of the nude mice in lead II position (right forelimb to left hind limb) (Menon *et al.*, 2018). Every recording lasted for at least 5 min. ECG recording speed was 50 mm/s and the voltage was 1 mV/cm. Noise was minimized by a digital filter. Analysis of ECG waves was done to calculate heart rate (beats/min), QRS duration (ms), QT interval (ms), which was corrected for heart rate using the Bazett formula [$QTc=QT/(\text{square root of RR interval})$], and PR interval (ms). For each parameter, measurements were done at three non-consecutive, randomly chosen points in every 5 min recording. The results are reported as mean of the three randomly selected segments.

Histological Examination

After taking ECG recording, all mice were killed with 3% pentobarbital sodium and the heart tissue were excised, weighed and placed in 10% buffered formalin solution for tissue fixation. After processing for paraffin sections of 5 µm thickness, tissue sections were stained with Hematoxylin and Eosin (H & E) and examined under a light microscope (magnification 400×).

Biochemical and Oxidative Stress Examination

After taking ECG recording, the samples of blood were taken and centrifuged. The obtained serum was

used for the analysis of biochemical parameters. The lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GPX) levels were detected by UV spectrophotometer or microplate reader at 440, 660, 532 and 550nm according to the instructions of the kits.

Western blot analysis

Western blot performed following the method used as described (Huang *et al.*, 2018). The tissues were lysed at 4 °C and then supernatants collected after centrifugation. The protein concentration was measured by BCA assay. Protein lysate was loaded on and separated by a 10% SDS-PAGE, and then transferred to polyvinylidene fluoride membrane. The membranes were probed with antibodies against caspase3, phosphorylated and total protein kinase A (PKA), phosphorylated and total p38 respectively. Signals were amplified and observed with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling, CA, U.S.A., 1:5000 dilution) and enhanced chemiluminescence. Densitometry was detected with an ECL Western Blot Detection System (4A Biotech, Beijing, China). All experiments were repeated at least three times.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. Results from six mice ($n = 6$) were

analyzed and expressed as means \pm SD. Statistical analysis was done using one-way ANOVA, and post hoc comparisons were carried out using Duncan's multiple-range test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Change in the body weight and heart weight

After modeling, behavioral analysis of nude mice in each group found that nude mice had reduced activity and weight loss. Body weight changes in nude mice are shown in Figure 1 & 2. In DOX group, body weight tended to decrease most notably compared with the other five groups after 15 days treatment. The co-treatment groups showed an increasing tendency of body weight, and the increasing tendency was higher than DOX group in the end (Figure 2). Description DOX model group modeling success ($p < 0.05$). At the end of the experiment, the abdominal aorta blood was taken and the heart was taken and weighed. As shown in Figure 3, the heart weight of DOX+SS group and DOX+P-PLN group (3 mg/kg) were higher compared with DOX group ($p < 0.05$), indicating that DOX has a certain degree of myocardial toxicity. SS and P-PLN have a certain role in alleviating DOX myocardial toxicity.

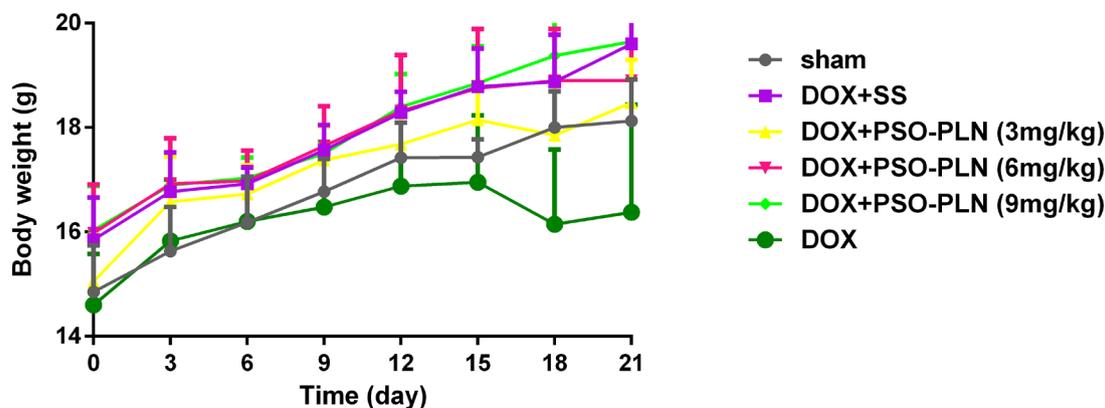


FIGURE 1 - Change in the body weight over 21 days in mice.

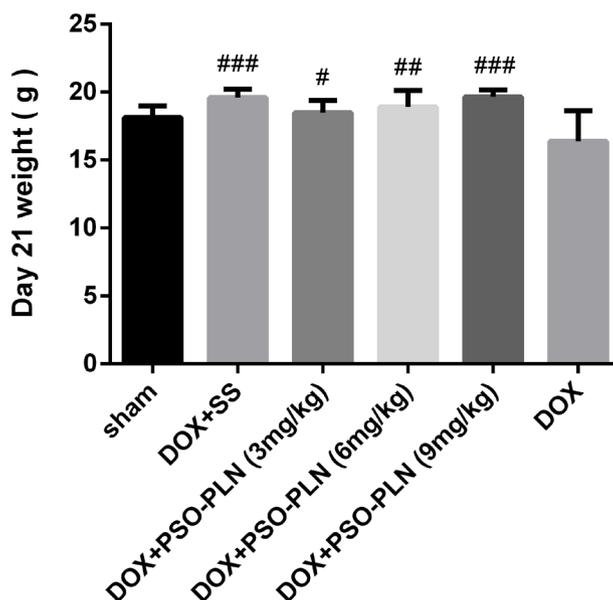


FIGURE 2 - Change in the body weight at day 21 in mice.

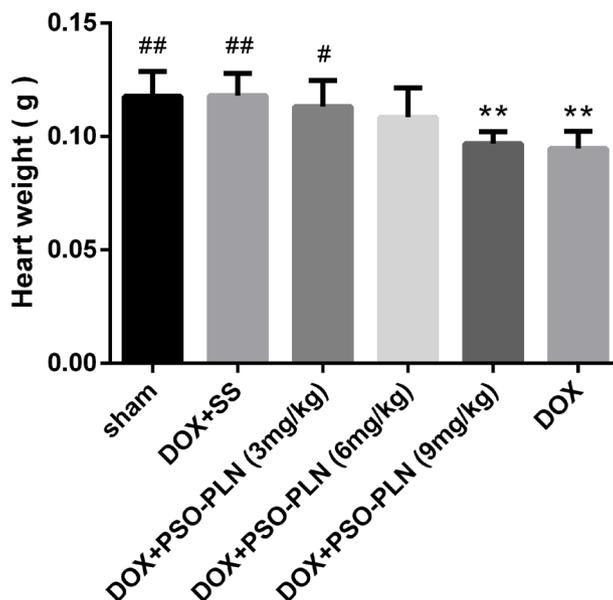


FIGURE 3 - Change in the heart weight over 21 days in mice.

Evaluation of ECG

Examples of ECG records from mice in each of the four groups at the end of the 21-day study period are given in Figure 4. DOX-treated mice showed significant prolongation of QRS duration after the 21 day treatment

period compared with the control group. After co-treatment with PSO-PLN and SS, this prolonging of the QRS complex was significantly attenuated compared with mice receiving DOX alone. No abnormal ECG parameters were observed in the controls.

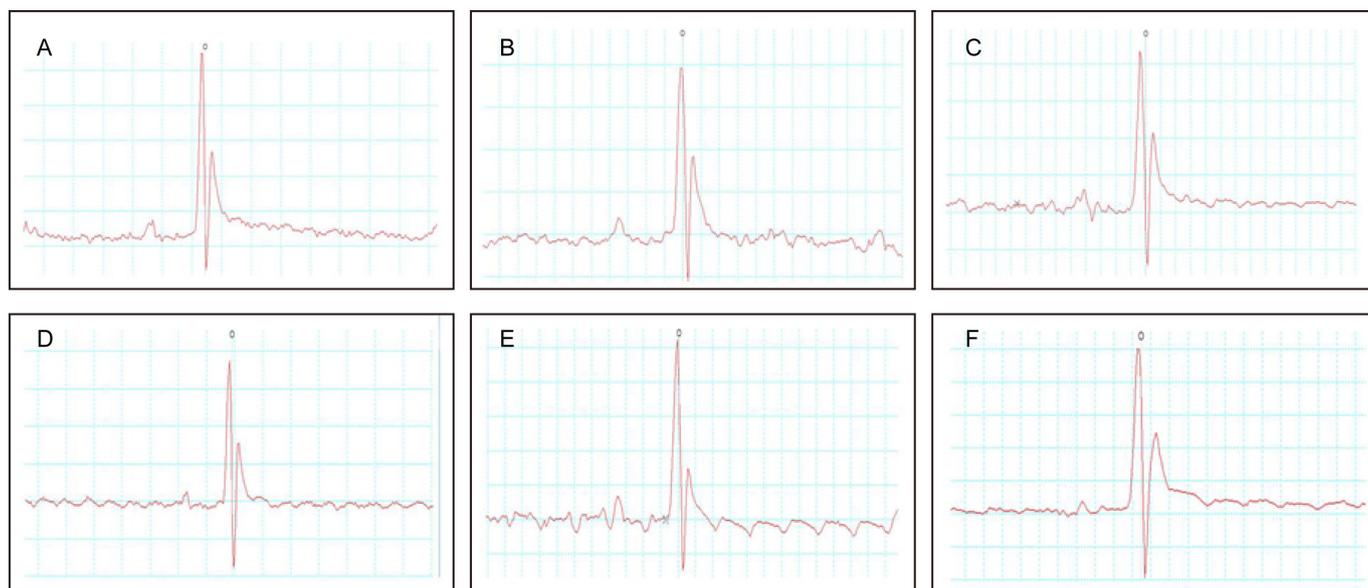


FIGURE 4 - Electrocardiogram recordings on day 21 day for mice injected intravenously on alternate days with : (A)sham, (B) DOX+SS, (C)DOX + PSO-PLN (3 mg/kg), (D)DOX+PSO-PLN (6 mg/kg), (E)DOX+PSO-PLN (9 mg/kg), (F)DOX.

Histological Examination

Figure 5 shows the representative examples of the histological appearance in the sham, DOX+SS, DOX+PSO-PLN (3, 6, 9 mg/kg), DOX groups after the 21-day treatment. Histological examination showed widespread marked structural abnormalities including less cardiomyocyte size, serious interstitial fibrosis,

cardiomyocyte necrosis, vacuolization and infiltration of mononuclear cells in DOX- exposed hearts. In the DOX+PSO-PLN (9 mg/kg), a small amount of cell fibrosis was observed. In contrast, necrotic cardiomyocytes, vacuolization and interstitial fibrosis were rare in DOX+SS, DOX+PSO-PLN (3 mg/kg) and DOX+PSO-PLN (6 mg/kg) groups. It indicates that SS and PSO-PLN (3, 6 mg/kg) can relieve the myocardial toxicity of DOX.

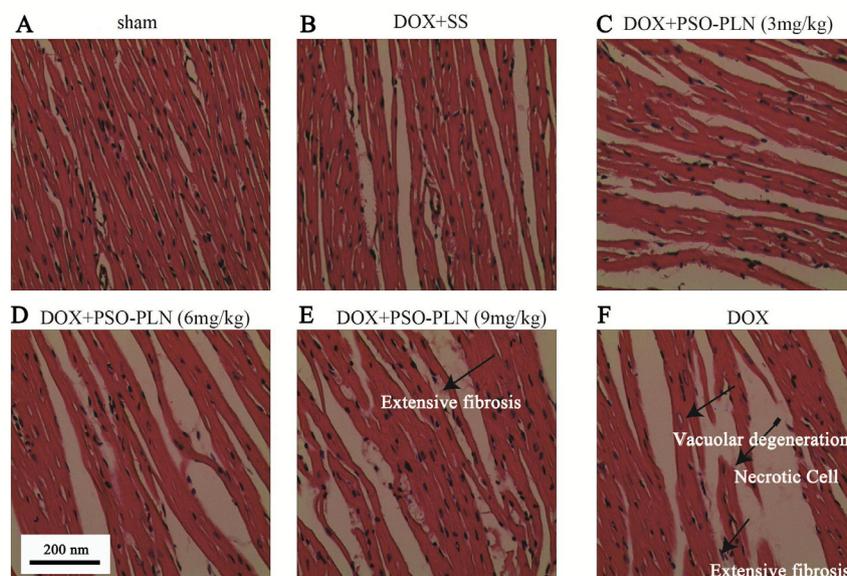


FIGURE 5 - Histological sections of the cardiac tissue on day 21 from mice treated with sham, DOX+SS, DOX + PSO-PLN (3 mg/kg), DOX+PSO-PLN (6 mg/kg), DOX+PSO-PLN (9 mg/kg), DOX.

Biochemical and Oxidative Stress Examination

Lactate dehydrogenase (LDH) and creatine kinase (CK) are important clinical markers of cardiac injury (Chen *et al.*, 2015). As expected, Figure 6 shows that serum levels of LDH and CK were significantly elevated in the DOX alone treated group as compared with the saline ($p < 0.05$). Treatment with PSO-PLN (3 mg/kg) significantly reduced their levels as compared with the DOX alone treated group ($p < 0.05$). Simultaneously, PSO-PLN (9 mg/kg) showed significantly increased the levels compared with the sham group ($p < 0.05$). And to confirm the induction of oxidative

stress by DOX, tissue lipid peroxidation, antioxidation and antioxidant enzymes were also evaluated. As shown in Figure 6, DOX group obviously increased serum levels of MDA content, and decreased the GSH content, GPX and SOD activities compared with sham ($p < 0.05$). As compared with DOX alone group, PSO-PLN (3 mg/kg) treatment lowered MDA levels ($p < 0.05$), retained GSH content, and recovered cardiac GPX and SOD activities ($p < 0.05$). While, the PSO-PLN (9 mg/kg) group had little therapeutic effect. Indicating that PSO-PLN (3 mg/kg) have a certain role in alleviating DOX induced cardiac injury and oxidative stress.

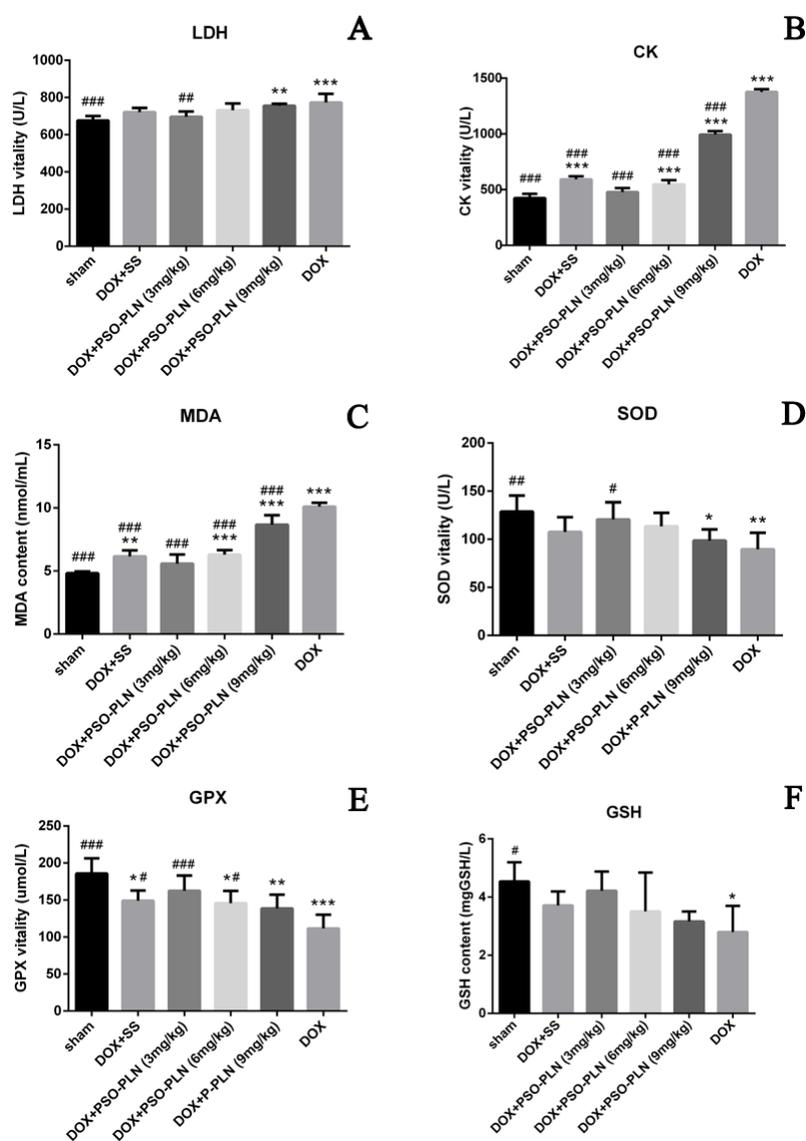


FIGURE 6 - Effect of PSO-PLN on serum levels of lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GPX) in DOX-treated mice.

Western blot analysis

Previous studies found that Cyclic Adenosine monophosphate-protein kinase A (cAMP-PKA) signaling pathway promoted cardiomyocyte survival (Maurice *et al.*, 2003). Other studies have shown that p38 mitogen-activated protein kinases (p38 MAPK) pathway is involved in the DOX-induced cardiac oxidative, inflammatory and apoptotic reactions (Guo *et al.*, 2013). Therefore, the

phosphorylation of PKA and p38, and their total protein levels were investigated by Western blotting. As shown in Figure 7, in the DOX treatment group, the expression of phosphor-PKA and PKA, show significantly decrease of about 0.71 and 0.90; the expression of phosphor-p38 and p38 show obviously increase of about 1.38 and 1.01 respectively, whereas PSO-SLN (3, 6 mg/kg) and SS co-treatment modulated the activation of both protein kinases that were elevated in the presence of DOX.

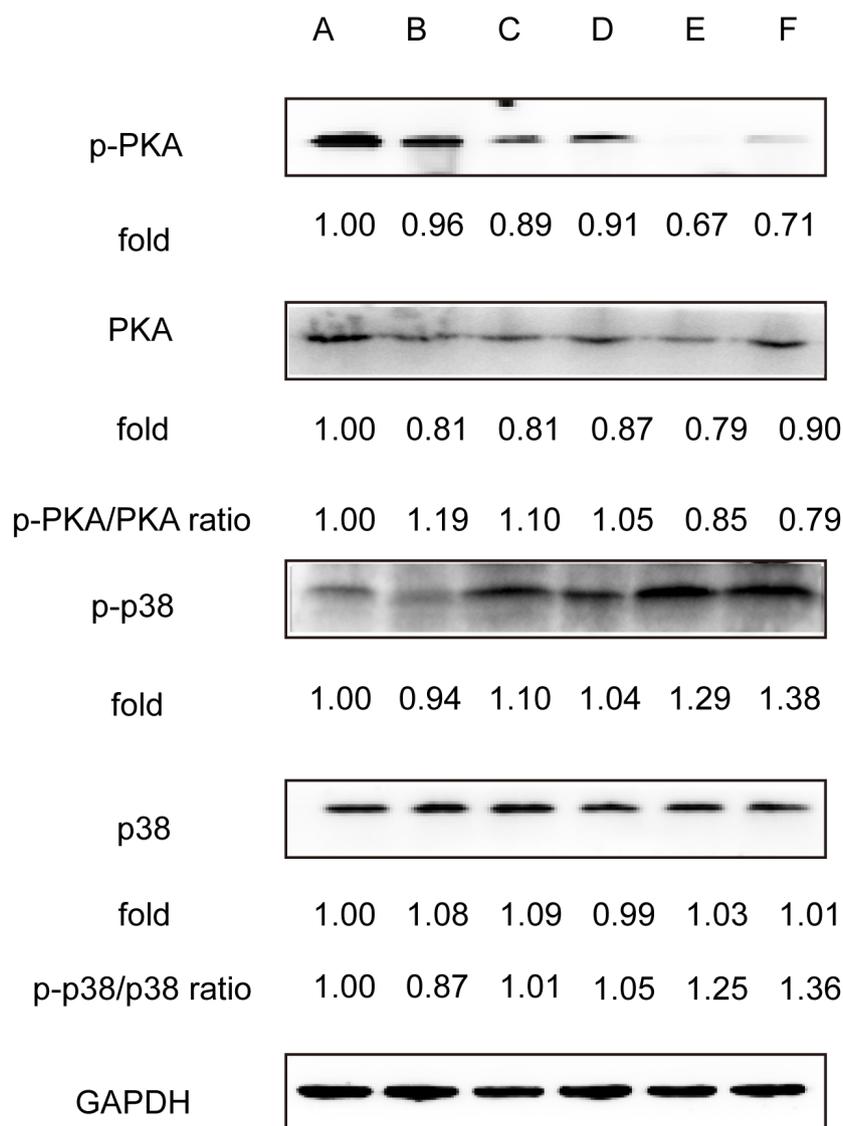


FIGURE 7 - Western blot analysis of activation of PKA and p38 in heart tissues of nude mice (A: sham; B: DOX+SS; C: DOX+PSO-PLN (3 mg/kg); D: DOX+PSO-PLN (6 mg/kg); E: DOX+PSO-PLN (9 mg/kg); F: DOX).

DISCUSSION

Cardiotoxicity, a major side effect of DOX, can be observed in clinical patients and animal studies. Multiple mechanisms are involved in DOX induced cardiomyopathy, such as the increase in cardiac oxidative stress and lipid peroxidation, and changes in adenylate cyclase activity leading to apoptosis and inflammation-related signaling pathway (Shi *et al.*, 2011; Hajra *et al.*, 2018). Our data indicated that co-treatment of PSO-PLN (3 mg/kg) with DOX for 21-days improved cardiac function during the DOX-induced cardiomyopathy, as demonstrated by improvements in body weight, heart weight, and in serum levels of LDH, CK, MDA, SOD, GSH, and GPX. The mechanism of PSO-PLN protecting myocardial cells may be related to decrease the level of oxidant stress. And a similar response has been reported in mice models, lowering the level of oxidative stress in myocardial cells may has a certain protective effect on injured myocardial cells (Lin, Yin, 2013; Patil, Balaraman, 2012).

The DOX-induced heart toxicity has been characterized by the oxidative stress in the heart tissue. In Figure 6, the data showed that SOD, GPX activities and GSH content were significantly decreased in the DOX-treated mice. Meanwhile, MDA content was significantly increased. MDA is overproduced because of an increase in free radicals, as a lipid peroxidation marker (Gawel *et al.*, 2004). SOD and GPX are important antioxidant enzymes which plays a predominant role in removing excess free radicals and hydroperoxides from the cell (Alam *et al.*, 2018). By catalyzing GSH to reduce hydroperoxide, GPX can effectively remove free radicals in organisms and thus protect cells from oxidative damage (Farah *et al.*, 2016). Antioxidant enzymes form the first line of defense against cardiac tissue damage, and an increased oxidative stress may be due to depletion of antioxidants as reported earlier (Chen *et al.*, 2015). This result supports the theory of reactive oxygen species (ROS). And the serum level of MDA was obviously decreased in the group of co-treatment PSO-PLN (3 mg/kg) compared with DOX alone treated, and with a concomitant rise in the activity of SOD, GPX and GSH content. These data indicating

that PSO-PLN (3 mg/kg) have a certain role in alleviating DOX induced cardiac injury and oxidative stress. The protection thus offered may be due to their antioxidant and ROS scavenging nature.

Western blotting results demonstrated that PSO-PLN (3, 6 mg/kg) can increase the expression of p-PKA and alleviate the DOX-induced cardiac cell death effect leading to a down-regulation of cardiomyocyte contractility via cAMP-PKA pathway (Kozubowski, Lee, Heitman, 2009). Many previous studies revealed that MAPKs play a crucial role in the development of hypertrophy processes such as inflammation and fibrosis (Liao *et al.*, 2001; Dhalla, Müller, 2010). Our present study showed that PSO-PLN down-regulated the expression of p-p38. It is possible that the inhibited effect of PSO-PLN on the DOX-induced cardiotoxicity was conducted via inactivating p38.

CONCLUSIONS

DOX-induced myocardial toxicity may limit its therapeutic use in clinic. The search for cardioprotective agents will continue to rely on increasing our understanding of the mechanisms of the DOX-induced cardiotoxicity and how to counteract and overcome it. Our data suggests that PSO-loaded polymer lipid nanoparticles (3 mg/kg) could ameliorate the doxorubicin-induced myocardial toxicity. It can also suggests the promising role of psoralen (PSO) as a cardioprotective agent against the DOX-induced cardiotoxicity. PSO is a potential antioxidant molecule that has a helpful effect on the heart against DOX. The application of nanotechnology is a novel strategy that may have much potential for protecting against doxorubicin-induced cardiotoxicity in clinical practice.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol for the study was approved by the College of Pharmacy, Jinan University. The Laboratory Animal Ethics Committee of Jinan University approved all protocols (date of approval, 10/03/2017; certification no. 20170310120637).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Erratum

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