Pakistan J. Zool., vol. 52(5), pp 1787-1793, 2020 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20200218020203

Effect of Pilose Antler Peptide on Doxorubicin-induced H9c2 Cells Injury via TGF-β/Smad/ERK Signaling Pathway

Yan Xu[1], Jia Zhou[1], Guangfu Lv[2], Yuexin Liu[1], Xintong Zhao[1], Xin Li[1], Doudan Ye[2], Xiaobo Qu[1,*] and Xiaowei Huang[1]*

[1]*School of Pharmaceutical Sciences, Changchun University of Chinese Medicine, Changchun 130117, China*
[2]*Ginseng Research Institute of Jilin Province, Changchun University of Chinese Medicine, Changchun 130117, China*

Article Information
Received 28 July 2019
Revised112 February 2020
Accepted 14 March 2020
Available online 15 May 2020

Authors' Contribution
YX, XQ and XH designed the study. XL and DY conceived and designed the study. YX, JZ and GL performed the experiments and analyzed the data. YL and XZ helped in microscopic examinations. All authors read and approved the final manuscript.

Key words
Doxorubicin, Cardiotoxicity, Pilose antler polypeptides, TGF-β / Smad / ERK pathway, H9c2 cells

ABSTRACT

Doxorubicin (DOX) is widely used clinically for the treatment of various malignant tumors, but it is accompanied by severe body toxic reactions, especially cardiac toxicity. This article explores the effects of pilose antler peptide (PAP) on DOX-induced myocardial injury and related mechanisms through in vitro experiments. Different concentrations of DOX were used to treat H9c2 cells for 6, 12, 24, and 48 h, respectively, to determine the IC_{50}. Different concentrations of PAP were treated to H9c2 cells for 24, 48, and 72 h to determine the effect of PAP on H9c2 cells. The experiment was divided into control group, DOX group, PAP group and DOX+PAP group. Lactate dehydrogenase (LDH) leakage and creatine kinase MB (CK-MB) level were used to detect cell viability, flow cytometry was used to detect cell cycle and apoptosis, and immunofluorescence was used to detect Bax and Bcl-2 expression. The protein levels of TGF-β / Smad / ERK signaling pathway were detected by Western blot. We found that PAP significantly increased cell viability after DOX-induced injury, reduced LDH, CK-MB levels, up-regulated Bcl-2 expression and down-regulated Bax expression levels. In addition, PAP can delay cell G2/M phase arrest, reduce apoptosis, significantly reduce TGF-β_1 protein levels and Smad2, Smad3, ERK phosphorylation levels. These results suggest that PAP can protect DOX-induced H9c2 damage by inhibiting the TGF-β / Smad / ERK signaling pathway.

INTRODUCTION

Cardiovascular disease is the most important disease that seriously threatens human life and health. Heart failure is a syndrome secondary to cardiovascular disease, which seriously affects the quality of life of patients. Studies showed that heart failure was often accompanied by myocardial apoptosis and myocardial fibrosis (Uchmanowicz *et al*., 2019).

Doxorubicin (DOX) is widely used in clinical treatment of various malignant tumors, such as acute leukemia, lymphoma, breast cancer and so on. But it has strong cardiotoxicity, and it is easy to accumulate in myocardial cells which makes myocardial tissue more susceptible to damage by DOX. In additon, DOX has a dose-dependent irreversible damage characteristic, which also increases the prevalence of cardiovascular disease in cancer surviving patients (Xu *et al*., 2019). However, related studies have used it to replicate animal models of heart failure, which can better simulate the state of myocardial apoptosis and myocardial fibrosis in heart failure (Xia *et al*., 2018). Nowadays, more and more traditional Chinese medicine and its extracts are being used in the treatment of cardiovascular diseases. Deer antler is a traditional Chinese medicine that has been used in China for more than 2000 years for the treatment of clinical heart disease in Chinese medicine. Pilose antler peptides (PAP) are a class of small protein substances extracted from pilose antler. It has been reported to have anti-inflammatory, anti-pulmonary fibrosis, and immunity enhancement effects, and there have been no reports of toxic reactions and side effects (Xu *et al*., 2018; Dong *et al*., 2018). This study observes the effect of PAP on DOX-induced H9c2 cell injury.

TGF-β / Smad / ERK signaling pathway is closely related to myocardial apoptosis or fibrosis. When the myocardium is damaged, the expression of TGF-β_1 increases, which regulates the downstream Smad and ERK proteins to conduct signals through phosphorylation and induce myocardial cell decay (Khalil *et al*., 2018; Pardali and Dijke, 2012; Jin and Yu, 2019).

In this study, we observed the effects of PAP on

* Corresponding author: 15948000740@163.com
0030-9923/2020/0005-1787 $ 9.00/0

DOX-induced H9c2 cells injury *in vitro* and explored its mechanism with the TGF-β / Smad / ERK signaling pathway.

MATERIALS AND METHODS

Materials

PAP was prepared by our lab (No.20190320). DOX was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Assay kits of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). All antibodies were obtained from Proteintech Group, Inc. (Wuhan, China).

H9c2 cell culture and CCK-8 assay

H9c2 cell lines were obtained from kindly provided by Stem Cell Bank, Chinese Academy of Science (Shanghai, China). Cells were maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with fetal bovine serum (FBS) (10%), penicillin (100 U/mL), and streptomycin (100 U/ml).

There were 6×10^3 cells/well seeded in 96-well plates and incubated for 24 h. The cells were treated with 0.1, 0.5, 1, 5, 10 and 20 μM of DOX for another 6, 12, 24, or 48 h. Cell viability was monitored using cell counting kit-8 according by the manufacturer's instructions. The absor bance was measured at 450 nm using a SynergyTM H1 instrument (BioTek, American). Effect of PAP on H9c2 cell was measured as above for another 24, 48, 72 h. Each experiment was performed at least three times under each corresponding experimental condition.

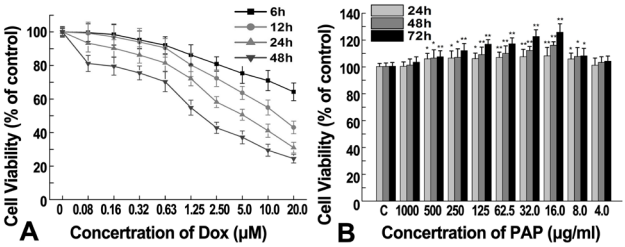

Fig. 1. Effects of different concentrations and time points of DOX and PAP on H9c2 cell viability using the CCK-8 assay. (A) DOX treatment concentrations were 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5, 10 and 20 μM, and DOX treatment time points were 6, 12, 24 and 48 h, respectively. (B) PAP treatment concentrations were 4.0, 8.0, 16.0, 32.0, 62.5, 125, 250, 500 and 1000 μg/ml, and PAP treatment time points were 24, 48 and 72 h, respectively.

As shown in Figure 1A and B, the CCK-8 assay demonstrated that H9c2 cell viability decreased with increasing DOX concentrations and times. However, different concentrations of PAP improved cell viability of H9c2 cell especially 16 μg/ml for 72 h. Therefore, treatment with DOX at 5 μM for 24 h was selected to establish the cardiotoxicity model in the present study.

Experimental grouping

The experimental groups were devided into 4 groups. Control group: cardiomyocytes grown in normal conditions; Dox group: cardiomyocytes treated with 5 μM DOX for 24 h; PAP group: cardiomyocytes treated with 16 μg/ml PAP for 72 h; DOX+PAP group: cardiomyocytes treated with 5 μM DOX for 24 h and 16 μg/ml PAP for 72 h.

Measurement of lactate dehydrogenase (LDH) leakage and CK-MB level in the culture medium

LDH and CK-MB levels in the supernatants after treatments were detected with LDH and CK-MB assay kit (Jiancheng, Nanjing, China). The experiment was performed according to manufacturer's specifications using a microplate reader (Bio-Rad 680, Hercules, CA, USA).

Assessment of apoptosis and cell cycle

1×10^6 cells were washed and resuspended in 1 ml of 1×binding buffer and centrifuged at 300 ×g for 10 min. Cell pellets were resuspended in 1×binding buffer, and added by 10 μl of Annexin V-FITC. After incubating with Annexin V for 15 min, cells were added by 5 μl of phycoerythrin solution immediately prior to analysis by flow cytometry.

Cells (1×10^6) were fixed with cold ethanol and rinsed with PBS for two times, then mixed with 100 μl PBS. Added with the final concentration of 50 g/ml propiridine iodide dye, which was reacted at 4°C in light for 30min. The cell cycle of H9c2 cells was detected by flow cytometry.

Detection of Bax and Bcl-2 activities

8×10^4 cells/well were seeded in 12-well plates and incubated for 24 h. After experimental grouping, 4% paraformaldehyde fixed for 30 min, 0.5% Triton X100 500 μl permeate for 15 min, 3% BSA 500 μl blocked for 30 min, antibodies of anti-Bax and anti-Bcl-2 (1:100) were added to each well and incubated at 4 °C overnight. Add secondary antibodies (1:100) and incubate for 1 h in the dark, add 500 μl DAPI to each well for 30 min, stain the slides with an anti-fluorescent quencher, and measure the fluorescence intensity with a laser confocal microscope. Washed 3 times with PBS for 5 min between steps.

Western blot assay

Total protein in H9c2 cells were extracted according to

the instructions on the protein extraction kits. Total protein was quantified with bicinchoninic acid (BCA) protein assay kits (Solarbio, Beijing, China). 20 μg protein were protein were loaded on an 8-12% SDS-polyacrylamide gel for electrophoresis. Blots were then transferred onto PVDF membranes and blocked in 5% skimmed milk at 25°C for 1 h on a shaking table. Membranes were incubated with the appropriate concentrations of specific antibodies at 4 °C overnight including TGF-β_1 (1:1,000), Smad2 (1:6,000), p-Smad2 (1:2,000), Smad3 (1:1,000), p-Smad3 (1:1,000), ERK (1:4,000), p-ERK (1:1,000) and anti-GAPDH (1:40,000). On the second day and after three rounds of washing with TBST, the membranes were incubated with secondary anti-rabbit (1:10,000) at 25°C for 1 h. Finally, the immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) kit on a gel imaging system.

Analysis of data

Data were represented as means ± standard deviations (SD), and obtained from at least three repeated experiments. Significant difference among data was assessed by one-way ANOVA followed by Tukey's post hoc test using Statistical Package for Social Science (SPSS) software (version 21.0; SPSS, USA), and a value of $P<0.05$ and $P<0.01$ were considered as statistically significant.

RESULTS

Effect of PAP on DOX-induced on H9c2 cells viability

To investigate cytotoxicity effects of DOX in cardiomyocytes, H9c2 cells viability were detected using the CCK-8 assay after exposed to different concentrations of DOX for 6, 12, 24, and 48 h. As shown in Figure 1A, DOX exhibited significant cytotoxic effects with an IC_{50} value of 5 μM at 24 h. On the contrary, H9c2 cell viability was improved with different time and concentration of PAP, especially 0.16 mg/ml at 72 h as shown in Figure 1B. In addition, PAP could enhance DOX-induced H9c2 cell viability, as shown in Figure 2. The results showed that PAP could promote survival of H9c2 cells induced by DOX. In order to determine the mechanism of effect, treatment with 5μM DOX for 24 h and 0.16 mg/ml PAP for 72 h were selected for the subsequent experiments.

Effects of PAP on DOX-induced LDH leakage and CK-MB level in H9c2 cells

LDH is an enzyme that exists in the cytoplasm and is released into the medium when the cell membrane is damaged (Tian *et al*., 2019). CK-MB which mainly distributes in the cardiomyocytes, is released extracellularly when myocardial damage occurs. LDH leakage and CK-MB level were further detected to illustrate cell death. As shown in Figure 3, LDH leakage and CK-MB level in the medium increased significantly after incubation with DOX for 24 h compared with control group ($P<0.01$). Treatment with PAP effectively suppressed the LDH leakage and CK-MB level compared with DOX group ($P<0.01$).

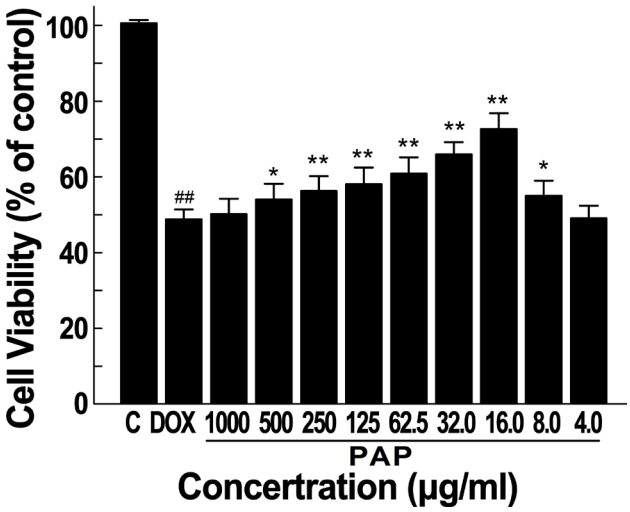

Fig. 2. Effect of PAP on H9c2 cells from DOX-induced cell death. PAP treatment concentrations were 4.0, 8.0, 16.0, 32.0, 62.5, 125, 250, 500, 1000 μg/ml for 72 h and DOX treatment with 5 μM for 24 h.

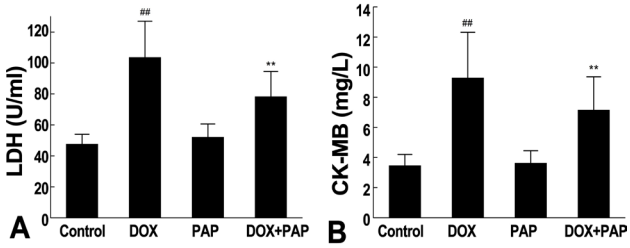

Fig. 3. Effects of PAP on DOX-induced LDH leakage and CK-MB level in H9c2 cells. (A) LDH leakage and (B) CK-MB level were detected in the cellular supernatant.

Effects of PAP on DOX-induced apoptosis and cell cycle in H9c2 cells

We detected the cell cycle and apoptosis by flow cytometry. The results showed that H9c2 cells in the DOX group were significantly apoptotic compared with control group, while the G2/M phase cells were significantly increased ($P<0.01$). However, there were no significant changes in the PAP group ($P > 0.05$). Compared with the DOX group, DOX+PAP group G2/M phase H9c2 cells arrest reduced and the number of apoptotic cells decreased significantly ($P<0.05$ or $P<0.01$), as shown in Figure 4.

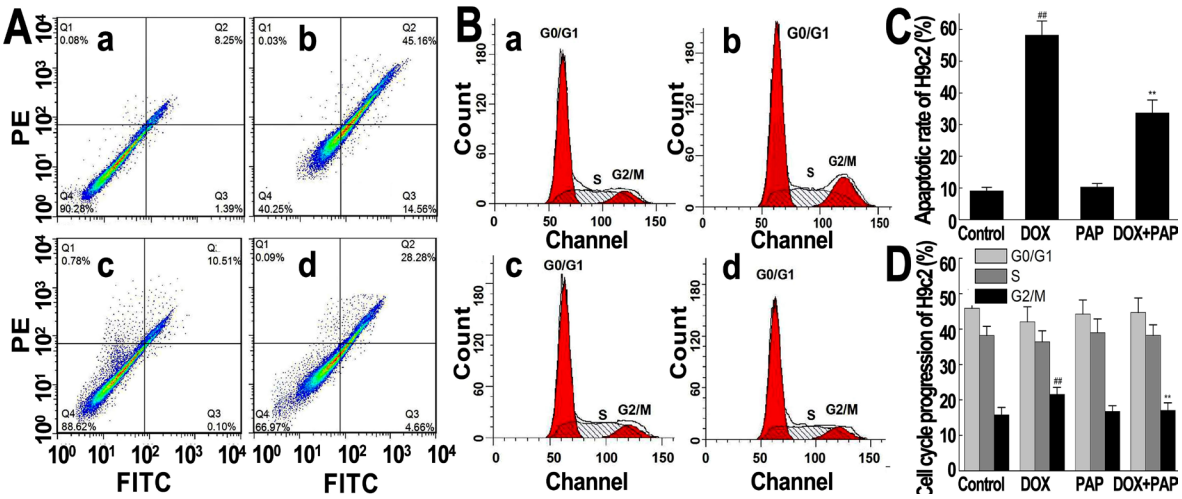

Fig. 4. Effects of PAP on DOX-induced Apoptosis and Cell Cycle in H9c2 cells. (A) Apoptosis and (B) Cell Cycle were detected by flow cytometry. (C) Cell Cycle progression and (D) apoptotic rate of H9c2 cells were presented in panel.

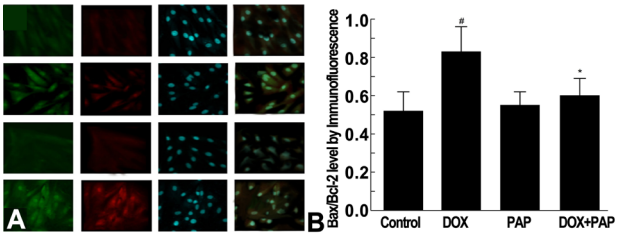

Fig. 5. Effects of PAP on DOX-induced Bax and Bcl-2 expression in H9c2 cells. Bax and Bcl-2 expression were detected by Immunofluorescence. (A) Fluorescence images of cells showed Bcl-2 immunofluorescence (green), Bax immunofluorescence (red) and DAPI staining (blue). (B) Bar image represents Bax / Bcl-2 expression level.

Effects of PAP on DOX-induced Bax and Bcl-2 expression in H9c2 cells

Bax and Bcl-2 are homologous water-soluble related proteins. Bcl-2 can inhibit apoptosis, however overexpression of Bax can antagonize the protective effect of Bcl-2 and make cells tend to die. Immunofluorescence results show that compared with the control group, the expression of Bcl-2 in the DOX group decreased, while the expression of Bax increased obviously, besides the number of living cells also decreased. The value of Bcl-2/Bax was significantly reduced ($P<0.01$). There was no significant change in the PAP group ($P > 0.05$). Compared with the DOX group, DOX + PAP group the expression of Bax decreased and Bcl-2 increased. The value of Bcl-2/Bax was significantly increased ($P<0.05$), as shown in Figure 5.

Effects of PAP on DOX-induced protein level in the TGF-β/SMAD/ERK pathway

TGF-β/SMAD/ERK pathway is a crucial mediator governing myocardial damage which we performed western blotting to determine the protein level. As shown in Figure 6, DOX induction caused an obvious elevation in the protein levels of TGF-β_1 compared to levels found in the control group ($P<0.01$). There were no significant changes in Smad2, Smad3 and ERK, whereas their phosphorylation protein levels increased significantly ($P<0.01$). With treatment of PAP their phosphorylation protein levels decreased significantly compared with the DOX group ($P<0.05$ or $P<0.01$).

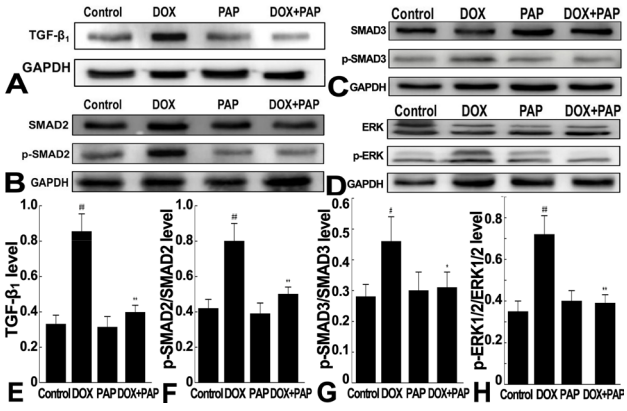

Fig. 6. Effects of PAP on DOX-induced protein level in the TGF-β/SMAD/ERK pathway. The protein levels of (A) TGF-β_1, (B) SMAD2, p-SMAD2, (C) SMAD3, p-SMAD3, (D) ERK and p-ERK were detected by western blot. Bar images represent (E) TGF-β2 (F) p-SMAD2/SMAD2, (G) p-SMAD3/SMAD3 and (H) p-ERK/ERK levels, respectively.

DISCUSSION

Myocardial injury caused by various reasons, as the disease continues and the course of the disease is chronic, will lead to myocardial fibrosis and local myocardial cell apoptosis, leading to gradual decline of cardiac function, and eventually induce cardiac dysfunction, arrhythmia and even heart failure. Heart failure is a serious and end stage of various heart diseases and the most important chronic cardiovascular disease of the 21st century. It has a high mortality rate and a poor prognosis (Li *et al*., 2018). According to statistics, there are more than 10 million heart failure patients worldwide, and the prevalence increases significantly with age.

DOX is a highly effective anthracycline chemotherapeutic drug, and it is also a first-line drug for the treatment of a variety of cancers in the clinic. However, its induced cardiotoxicity has limited its application. Studies have reported that the cardiac tissue affinity of DOX is obvious. Higher than other tissues, and with the accumulation of DOX dose, it has a dose-dependent irreversible damage characteristic, such as increased myocardial fibrosis area and increased myocardial cell apoptosis (Abuosa *et al*., 2018; Wena *et al*., 2019). Based on this, DOX is also widely used in the replication of myocardial injury and animal models of myocardial fibrosis. At present, the mechanism of DOX cardiotoxicity mainly includes the production of a large number of reactive oxygen free radicals (ROS), the abnormal increase of intracellular calcium content, and the damage of cell structure and functional metabolism, cell autophagy or apoptosis. Wait. Pilose antler has been used in China for more than 2,000 years as a traditional Chinese medicine and has been used clinically for the treatment of heart disease (Xu *et al*., 2020; Imam *et al*., 2018). PAP is a small protein substance existing in pilose antler, which has pharmacological effects such as anti-inflammatory, anti-oxidant, strengthening the immune system and anti-toxic (Bai *et al*., 2017; Ni *et al*., 2019). In this study, H9c2 cells were used as the research object to observe the protective effect and mechanism of pilose antler peptide on adriamycin-induced myocardial injury through in vitro experiments.

CCK-8 experiments showed that the IC_{50} of H9c2 cells injury induced by DOX was 24 h and 5 μM, and PAP was non-toxic to H9c2 cells and had a certain effect of promoting proliferation. The optimal proliferation conditions were 16 μg/ml for 72 h. In summary, based on the above results, the experiment is divided into four groups, namely control, DOX, PAP, and DOX+PAP. The destruction of cell membrane structure caused by apoptosis or necrosis will cause enzymes in the cytoplasm to be released into the culture medium, including LDH, which has a relatively stable enzyme activity. The results of LDH research show that PAP can significantly reduce LDH leakage caused by DOX and reduce apoptosis. CK-MB mainly exists in cardiomyocytes. When cardiomyocyte injury occurs, cardiomyocytes undergo apoptosis, membrane integrity and permeability change, and macromolecules in cells escape. These macromolecular substances are therefore called myocardial injury markers. CK-MB is by far the best serum enzyme indicator for the diagnosis of myocardial infarction and has been widely used in clinical practice (Dai *et al*., 2018). CK-MB test results showed that PAP can reduce the apoptosis of H9c2 cells caused by DOX, and reduce the content of CK-MB in the supernatant. To further detect the occurrence of apoptosis, we used immunofluorescence technology to further detect the expression of apoptotic proteins Bax and Bcl-2. We found that compared with the control group, Bax fluorescence intensity of H9c2 cells in the DOX group increased, and Bcl-2 expression decreased. After the administration of PAP, the fluorescence intensity of Bcl-2 was significantly higher than that of Bax, the ratio of Bax / Bcl-2 was significantly reduced, and the apoptosis was reduced. Studies have shown that when heart failure or myocardial fibrosis occurs, cardiomyocytes undergo irreversible damage and block G2/M phases (Liu *et al*., 2016). Further exploring the mechanism of the protective effect of PAP on DOX-induced H9c2 apoptosis, we found that DOX-induced H9c2 cells increased apoptosis and the cells in the G2/M phase increased significantly and blocked, which also meant that some cells had irreversible Apoptosis, and PAP can reduce the number of H9c2 cells in G2/M phase to a certain extent, and the cell cycle tends to be normal. ERK is the core of the signal network that regulates cell growth, development, and division, and can regulate the transformation of cells to G2/M phase and the formation of spindles (Ranjani *et al*., 2015). The results showed that PAP reduced DOX-induced H9c2 cell ERK protein phosphorylation, inhibited H9c2 cells from blocking at the G2/M phase, and significantly reduced apoptosis.

It has been reported that when myocardial injury or heart failure is induced by doxorubicin, myocardial cell apoptosis is often accompanied by myocardial fibrosis, and the TGF-β / SMADs signaling pathway is closely related to tissue fibrosis (Wang *et al*., 2019). Among them, Smads protein is the main effector molecule in the middle and lower reaches of the signaling pathway, and TGF-β receptor signals are transmitted through phosphorylated Smads proteins to regulate the expression of related genes and promote the development of fibrosis,

especially the levels of Smad2 and Smad3 proteins (Hanna and Frangogiannis, 2019; Zeng *et al*., 2019). The results show that PAP can significantly reduce the expression of TGF-β_1 in H9c2 cells induced by DOX, and the levels of phosphorylation of Smad2 and Smad3.

CONCLUSIONS

In summary, PAP can inhibit cell G2/M phase arrest by reducing the phosphorylation level of ERK protein, reduce H9c2 apoptosis caused by DOX, and reduce the expression level of TGF-β_1 in cells, as well as the phosphorylation levels of Smad2, Smad3 and ERK which reduced degree of myocardial fibrosis.

ACKNOWLEDGEMENT

We acknowledge the financial supports of the National Natural Science Foundation of China under Grant No. 81774147 and Education Department of Jilin Province Project under Grant No. JJKH20181232KJ.

Statement of conflict of interest

The authors Have declared no conflicts of interest.

REFERENCES

Abuosa, A.M., Elshiekh, A.H., Qureshi, K., Abrar, M.B., Kholeif, M.A., Kinsara, A.J., Andejani, A., Ahmed, A.H. and Cleland, J.G.F., 2018. Prophylactic use of carvedilol to prevent ventricular dysfunction in patients with cancer treated with doxorubicin. *Indian Heart J.*, **70**: S96-S100. https://doi.org/10.1016/j.ihj.2018.06.011

Bai, L., Shi, W., Liu, J., Zhao, X., Zhang, Y., Zhou, Z., Hou, W. and Chang, T., 2017. Protective effect of pilose antler peptide on cerebral ischemia/reperfusion (I/R) injury through Nrf-2/OH-1/NF-kappa B pathway. *Int. J. Biol. Macromol.*, **102**: 741-748. https://doi.org/10.1016/j.ijbiomac.2017.04.091

Dai, G.F., Wang, Z. and Zhang, J.Y., 2018. Octreotide protects doxorubicin-induced cardiac toxicity via regulating oxidative stress. *Eur. Rev. Med. Pharmacol.*, **22**: 6139-6148.

Dong, Y., Liu, L., Shan, X., Tang, J., Xia, B., Cheng, X., Chen, Y. and Tao, W., 2018. Pilose antler peptide attenuates LPS-induced inflammatory reaction. *Int. J. Biol. Macromol.*, **108**: 272-276. https://doi.org/10.1016/j.ijbiomac.2017.11.176

Hanna, A. and Frangogiannis, N.G., 2019. The role of the TGF-beta superfamily in myocardial infarction. *Front. Cardiovasc. Med.*, **6**: 140. https://doi.org/10.3389/fcvm.2019.00140

Imam, F., Al-Harbi, N., Al-Harbi, M., Ansari, M.A., Al-Asmari, A.F., Ansari, M.N., Al-Anazi, W., Bahashwan, S., Almutairi, M., Alshammari, M., Khan, M.R., Alsaad, A.M. and Alotaibi, M.R., 2018. Apremilast prevent doxorubicin-induced apoptosis and inflammation in heart through inhibition of oxidative stress mediated activation of NF-kappa B signaling pathways. *Pharmacol. Rep.*, **70**: 993-1000. https://doi.org/10.1016/j.pharep.2018.03.009

Jin, H. and Yu, J., 2019. Lidocaine protects H9c2 cells from hypoxia-induced injury through regulation of the MAPK/ERK/NF-kappa B signaling pathway. *Exp. Ther. Med.*, **18**: 4125-4131. https://doi.org/10.3892/etm.2019.8055

Khalil, H., Kanisicak, O., Prasad, V., Correll, R.N., Fu, X., Schips, T., Vagnozzi, R.J., Liu, R., Huynh, T., Lee, S.J., Karch, J. and Molkentin, J.D., 2017. Fibroblast-specific TGF-beta-Smad2/3 signaling underlies cardiac fibrosis. *J. clin. Invest*, **127**: 3770-3783. https://doi.org/10.1172/JCI94753

Li, W., Zhang, X., Wu, R., Song, Y. and Xie, M., 2018. Ganoderma atrum polysaccharide improves doxorubicin-induced cardiotoxicity in mice by regulation of apoptotic pathway in mitochondria. *Carbohyd. Polym.*, **202**: 581-590. https://doi.org/10.1016/j.carbpol.2018.08.144

Liu, P., Sun, Y., Qiu, G., Jiang, H. and Qiu, G., 2016. Silencing of TBX20 gene expression in rat myocardial and human embryonic kidney cells leads to cell cycle arrest in G2 phase. *Mol. med. Rep.*, **14**: 2904-2914. https://doi.org/10.3892/mmr.2016.5660

Ni, Y., Wang, Z., Ma, L., Yang, L., Wu, T. and Fu, Z., 2019. Pilose antler polypeptides ameliorate inflammation and oxidative stress and improves gut microbiota in updates hypoxic-ischemic injured rats. *Nutr. Res.*, **64**: 93-108. https://doi.org/10.1016/j.nutres.2019.01.005

Pardali, E. and Dijke, P., 2012. TGF beta signaling and cardiovascular diseases. *Int. J. biol. Sci.*, **8**: 195-213. https://doi.org/10.7150/ijbs.8.195

Ranjani, J., Pushpanathan, M., Mahesh, A., Niraimathi, M., Gunasekaran, P. and Rajendhranet, J., 2015. Pseudomonas aeruginosa PAO1 induces distinct cell death mechanisms in H9C2 cells and its differentiated form. *J. Basic Microbiol.*, **55**: 1191-1202. https://doi.org/10.1002/jobm.201500037

Tian, E., Hu, W., Miao, Y., Muhammad, I., Zhang, L., Xia, C., Ding, L., Zhang, Q., Li, R., Chen, C. and Li, J., 2019. Preventive effects of nerve growth factor

against colistin-induced autophagy and apoptosis in PC12 cells. *Toxicol. Mech. Method*, **29**: 177-186. https://doi.org/10.1080/15376516.2018.1534298

Uchmanowicz, I., Nessler, J., Gobbens, R., Gackowski, A., Kurpas, D., Straburzynska-Migaj, E., Kałuzna-Oleksy, M. and Jankowska, E.A., 2019. Coexisting frailty with heart failure. *Front. Psychol.*, **10**: 1-9. https://doi.org/10.3389/fphys.2019.00791

Wang, S., Li, D. and Yuan, Y., 2019. Long-term moderate intensity exercise alleviates myocardial fibrosis in type 2 diabetic rats via inhibitions of oxidative stress and TGF-beta 1/Smad pathway. *J. pharmacol. Sci.*, **69**: 861-873. https://doi.org/10.1007/s12576-019-00696-3

Wen, J., Wang, J., Li, P., Wang, R., Wang, J., Zhou, X., Zhang, L., Wei, S., Cai, H. and Zhao, Y., 2019. Protective effects of higenamine combined with [6]-gingerol against doxorubicin-induced mitochondrial dysfunction and toxicity in H9c2 cells and potential mechanisms. *Biomed. Pharmacother.*, **115**: 1-12. https://doi.org/10.1016/j.biopha.2019.108881

Xia, P., Liu, Y., Chen, J., Coates, S., Liu, D. and Cheng, Z., 2018. Inhibition of cyclin-dependent kinase 2 protects against doxorubicin-induced cardiomyocyte apoptosis and cardiomyopathy. *J. biol. Chem.*, **293**: 19672-19685. https://doi.org/10.1074/jbc.RA118.004673

Xu, C., Liu, C. and Zhang, D., 2020. MicroRNA-22 inhibition prevents doxorubicin-induced cardiotoxicity via upregulating SIRT1. *Biochem. biophs. Res. Commun.*, **521**: 485-491. https://doi.org/10.1016/j.bbrc.2019.10.140

Xu, L., Yan, L. and Tao, W., 2018. Pilose antler peptide attenuates high-fat-dietinduced liver injury. *Toxicol. Mech. Method*, **28**: 279-285. https://doi.org/10.1080/15376516.2017.1402978

Xu, S., Wang, Y., Yu, M., Wang, D., Liang, Y., Chen, Y., Liao, C., Xie, Z., Zhao, B., Han, J., Duan, Y. and Yang, X., 2019. LongShengZhi capsule inhibits doxorubicin-induced heart failure by anti-oxidative stress. *Biomed. Pharmacother.*, **123**: 1-9. https://doi.org/10.1016/j.biopha.2019.109803

Zeng, Z., Wang, Q., Yang, X., Ren, Y., Jiao, S., Zhu, Q., Guo, D., Xia, K., Wang, Y., Li, C. and Wang, W., 2019. Qishen granule attenuates cardiac fibrosis by regulating TGF-beta/Smad3 and GSK-3 beta pathway. *Phytomedicine*, **62**: 1-12. https://doi.org/10.1016/j.phymed.2019.152949