H3K9ac modification was involved in doxorubicin induced apoptosis by regulating Pik3ca transcription in H9C2 cells

Liu Yang, Yang Yu, Ge Tian, Hanyu Deng, Bo Yu

PII: S0024-3205(21)00092-8

DOI: https://doi.org/10.1016/j.lfs.2021.119107

Reference: LFS 119107

To appear in: *Life Sciences*

Received date: 24 October 2020

Revised date: 18 December 2020

Accepted date: 21 December 2020

Please cite this article as: L. Yang, Y. Yu, G. Tian, et al., H3K9ac modification was involved in doxorubicin induced apoptosis by regulating Pik3ca transcription in H9C2 cells, *Life Sciences* (2018), https://doi.org/10.1016/j.lfs.2021.119107

H3K9ac modification was involved in doxorubicin induced apoptosis by regulating Pik3ca transcription in H9C2 cells

Liu Yang, Yang Yu, Ge Tian, Hanyu Deng, Bo Yu[1]

1.Department of Cardiology, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning, 110001, P.R China.

2.Department of Cardiology, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning, 121001, P.R China.

Abstract

Aim: We evaluated the effect of H3K9ac modification on the transcriptional level of Pik3ca and the apoptosis induction effects of Pik3ca in the PI3K/AKT pathway in rat H9C2 cells.

Main methods: H9C2 cells were cultured with 1uM doxorubicin for 8h. The acetylation level of histone H3K9 in the transcriptional initiation area of Pik3ca was determined by CHIP-seq. The enrichment of mRNA fragment of Pik3ca transcription initiation region by H3K9ac antibody was detected by CHIP-qPCR, and the expression of Pik3ca was detected by real-time Polymerase Chain Reaction(rt-PCR) and western blot. The transcription efficiency of Pik3ca siRNA was detected by immunofluorescence and western blot. Cell Counting Kit8(CCK8) was used to detect the cell activity and flow cytometry was used to detect the apoptosis rate. Western blot was applied to assess the protein expression level of PI3K, P-AKT, AKT, Bcl2, BAX and cleaved-caspase3 in H9C2 cells.

Key findings: In doxorubicin-inducedH9C2 cells, the acetylation levels of histone H3K9 in the Pik3ca transcriptional initiation region significantly increased and promoted the transcription of Pik3ca. After knockdown of Pik3ca, the PI3K/AKT signaling pathway was inhibited, and the protein expression of Bcl2 was increased, the protein expression of BAX and cleaved-caspase3 were decreased. PI3K/AKT pathway activator partially reversed the effect of silencing Pik3ca.

Significance: In summary, the elevated H3K9ac level in the Pik3ca transcriptional initiation region promoted the transcription of Pik3ca, and Pik3ca promoted doxorubicin induced apoptosis in H9C2 cells by activating the PI3K/AKT pathway, providing a new theoretical basis for the study of doxorubicin cardiomyopathy.

Keywords: H3K9ac, Pik3ca, PI3K/AKT, Doxorubicin cardiotoxicity, Apoptosis

1. Introduction

Doxorubicin is an anti-tumor drug with strong anti-tumor activity, but its strong cardiotoxicity greatly limits the clinical application of this highly effective antitumor drug[1]. The continuous accumulation of doxorubicin dose will cause irreversible heart damage and eventually lead to heart failure. Previous studies have shown that doxorubicin can induce apoptosis in H9C2 cardiomyocytes through mitochondrial injury[2], endoplasmic reticulum stress[3], autophagy, and oxidative stress[4] etc. New research has found that doxorubicin can also inhibit tumor development by interfering with gene transcription[5].

[1] Corresponding author at: Department of Cardiology, The First Affiliated Hospital of China Medical University, No.155 Nanjing North Stress, Heping District, Shenyang, Liaoning, 110001, P.R China. E-mail address: ybdyybdy@163.com

However, the current research mechanisms are not very clear. Finding therapeutic for doxorubicin-induced cardiomyopathy is still a huge challenge.

Epigenetics is a heritable way of changing gene expression without changing DNA sequences, mainly including DNA methylation, histone modification (acetylation, methylation, ubiquitination), etc. [6]. Epigenetic modification is a reversible change initiation mainly by changes in the extracellular environment[7]. In recent years, studies have found that epigenetic modification is closely related to gene transcription. Chu, Chun Hsien et al. finds that in ANG-Ⅱ-induced hypertrophic cardiomyopathy, acetylation of histone H3, H4 promotes gene transcription, while DNA methylation inhibits gene transcription[8], which is consistent with previous results[9, 10]. Histone acetylation plays an increasingly important role in promoting gene transcription. There are many modification sites for histone acetylation, among which histone 3 and histone 4 are the most closely related to gene transcription[11]. In yeast, whole-genome CHIP-chip analysis shows that acetylation of histone 3 at lysine 9 and 14 sites has a stronger correlation with gene transcription than acetylation of histone 4 at lysine 5, 8, 12, 16 sites. The peak value of H3K9ac in gene transcription initiation region is more significant than that of H3K14ac[12], suggesting that H3K9ac may promote gene transcription more significantly than H3K14ac. H3K9ac is found to promote the expression of oncogene HIF-1α in breast cancer[13]. In mouse embryonic myocardial, H3K9ac mediated by P300 can promote the expression of GATA4 transcription factor[14]. Since doxorubicin can change gene transcription, acetylation of histone H3K9 has a regulatory effect on gene transcription. In order to verify the relationship between doxorubicin induced H9C2 cells and H3K9ac, H3K9ac antibody was used in this study to conduct CHIP-seq sequencing in doxorubicin induced H9C2 cells model. The analysis showed that the acetylation level of the genome changed significantly before and after doxorubicin intervention. For doxorubicin-induced changes in gene expression level, Pik3ca was finally determined by comparing the sequence of transcription initiation region recognized by H3K9ac antibody.

In this study, the expression of Pik3ca in doxorubicin-induced apoptosis in H9C2 cells was up-regulated, and apoptosis of H9C2 cells was changed after Pik3ca was silenced. In conclusion, H3K9ac modification was involved in doxorubicin induced apoptosis by regulating Pik3ca transcription in H9C2 cells. In this study, on the mechanism of doxorubicin-induced H9C2 cell apoptosis, Pik3ca was proved to be related to the PI3K/AKT pathway.

2. Materials and Methods

2.1 Cell culture and treatment

H9C2 cells was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in an incubator of 5% CO_2 at 37℃. After the cells grew to 80%-90%, 1uM doxorubicin was added to culture the cells for 8h for subsequent experimental operations.

2.2 Chromatin immunoprecipitation (CHIP)-seq.

The H9C2 cells were fixed in 40% formaldehyde for 10min, and 2.5M glycine was added to stop the crosslinking reaction. After the cells were lysed with cell lysis buffer, the nuclei were collected by centrifugation at 2000r for 5 min. The chromatin DNA was lysed by Sonic cracking method. Extract of DNA using phenol-chloroform method. Preparation

of high-throughput DNA sequencing libraries with Ⅰllumina V3 kit (catalog number: ND607 Vazyme), and library products corresponding to 200-500bps were enriched, quantified and sequenced on Novaseq 6000 sequencer (Ⅰllumina) with PE 150 model. Raw sequencing data was first filtered by Trimmomatic (version 0.36), low-quality read2 were discarded and the reads contaminated with adaptor sequences were trimmed. The clean reads were used for protein binding site analysis. The RSeQC (Version 2.6) was used for reads distribution analysis. The MACS2 software (Version2.1.1) was used for peak calling. The bedtools (Version 2.25.0) was used for peaks annotation and peak distribution analysis. The Homer (Version 4.10) was used for motifs analysis with a corrected P-value cutoff of 0.05 to judge statistically significant enrichment. CHIP-seq experiment and high through-put sequencing DNA data analysis were conducted by Seqhealth Technology Co, LTD (Wuhan, China).

2.3 Real- time Polymerase Chain Reaction (rt-PCR)

The H9C2 cells were inoculated into a six-well plate and the corresponding intervention factors were added. When the cells grew to about 90%, total RNA was extracted with TRIZOL reagent (Invitrogen, USA), and the optical density (OD) value was measured at the wavelength of 260nm with a visible spectrophotometer (NanoDrop Technologies Inc, Wilmington, USA), namely the concentration of total RNA. RNA was reverted to CDNA using a reverse transcription kit (TAKARA, Japan) with a total volume of 20ul at 37℃ for 15min, 85℃ for 5s, 4℃ for +∞. According to the instructions of the amplification kit(Takara, Japan), the amplification was carried out under the reaction conditions of 95℃ for 30s, 95℃ for 5s and 60℃ for 34s. The total reaction system was 20ul, 40 cycles in total. Primer sequences were as follows: for Pik3ca: Forward 5'-aggatgcccaacttgatgctgatg-3', Reverse 5'-ccgttcatatagggtgtcgctgtg-3'. For GAPDH: Forward 5'-gacatgccgcctggagaaac-3', Reverse 5'-agcccaggatgccctttagt-3'. CHIP-QPCR Pik3ca: Forward 5'-ggcttgagccttgagtaca-3', Reverse 5'-accagaagcgaggtgaacct -3'. Pik3ca siRNA: 5'-cccattcttctctatatca-3'.

2.4 Transfection

The cells were inoculated into a 6-well plate, and the supernatant was discarded after the cells grew to about 50%-60%. According to the reagent specification, using Pik3ca siRNA reagent (RIBO BIO Technology through, China) and Lipofectamine 2000 (Invitrogen, USA) mixed into OPTI - MEM medium cultured cells for 24h. After 24h, the supernatant was discarded and the culture was continued in complete medium containing serum. According to groups, PI3K/AKT activator 740Y-P (MedChemExpress, Monmouth Junction, NJ, USA) was added to culture medium to culture the cells for 24h, and then cells were collected for detection.

2.5 Immunofluorescence Assay (IF)

The cells were divided into three groups, namely the control (Con) group, the silent Pik3ca (siPik3ca) group and the negative control (NC) group. The cells were then evenly spread in a six-well plate containing a cover glass. When the cells density reached 50%-60%, transfection reagent was added and cultured cells for 6 hours. The transfection reagent was replaced with a complete medium for further culture until the cell density reached above 90%. The cell supernatant was discarded and washed with PBS for three times. Fix the cells with 4% paraformaldehyde for 15 minutes and washed with PBS three

times. Cells were incubated with 0.2%Trition X-100 at room temperature for 20 minutes and washed with PBS for three times. Removed the cover glass from the six-well plate and place it on the slide. Used a Pap pen to circle the cell area. Added normal goat serum and seal at room temperature for 30 minutes. After the goat serum was discarded, the diluted primary antibody was added and incubated at 4℃ in a wed box overnight. On the second day, the primary antibody was removed and PBS was washed three times. Added fluorescent secondary antibody and incubate in dark for 1h. Washed with PBS for three times and added DAPI staining for 3 minutes for detection.

2.6 Western Blot

In the six-well plate, the cells grew to 90 percent after intervention and were lysed with RIPA lysate (Beyotime, Biotechnology, China) and the total protein of the cells was extracted. The proteins were separated by gel electrophoresis with 10% SDS polyacrylamide gel and transferred to PVDF membrane. Then blots were sealed in 5% skim milk for 1h, washed with 1× TBST for 3 times, and finally incubated with primary antibody at 4℃ overnight. On the second day, the blots were washed three times with 1× TBST, 15min each time. The blots were incubated with horseradish peroxidase labeled secondary antibody for 1h at room temperature, and then washed three times with 1× TBST. Finally, the luminescence was performed with enhanced chemiluminescence (ECL, Cytiva, Shanghai, China). The blots were detected using the ChemiDoc XBS imaging system. Rabbit anti-Pik3ca antibody, anti-P-AKT antibody, anti-AKT antibody and anti-cleave-caspase3 antibody coming from Cell Signaling Technology. Rabbit anti-PI3K antibody and anti-bcl2 antibody come from Wanleibio (Shenyang, China). Rabbit anti-BAX antibody comes from Immunoway Biotechnology. Anti-GAPDH antibody comes from Cell Signaling Technology.

2.7 Annexin V-FITC/PI Assay

The cells were inoculated onto a six-well plate and grown to about 80% for transfection. Different interventions with doxorubicin and activator were added. Collect the cell supernatant. Accutase was used to digest the cells and collect the cells. After centrifugation at 1000rpm for 5 minutes, the supernatant was discarded and the cells were washed twice with precooled PBS. At the same time, three tubes of cells which were resuspended by adding 500UL typical Apoptosis Positive Control Solution were used to adjust the instrument parameters. Three tubes of cells put on the ice and incubated for 30min, followed by the discard of supernatant. Finally, each group cells were resuspended with 500ul 1×Binding Buffer. Annexin V-FITC 5UL and PI 10UL were added for each tube, BD FACSCanto flow cytometry analysis was performed after incubation at room temperature and dark for 5min. Annexin V-FITC/PI apoptosis kit comes from MULTI SCIENCES (Hangzhou, China).

2.8 Cell Viability Assay

Cell viability was measured using the Cell Counting Kit8 kit (APEBIO, USA). After the cells counting, the cells were laid evenly in the 96-well plate. The number of cells in each well was 5000, and the medium was 100ul/ well. After 24h of culturing, the intervention factors were added to each group of cells. The cells of the control group were cultured in a complete medium, cells in the experimental group were cultured with doxorubicin, while the blank control group contained no cells but doxorubicin, which was used to exclude the

interference of reagents on the cell activity. Finally, 10uL CCK8 reagent was added to the cells of each well and incubated in the incubator for 1h. The absorbance of each well was detected by a marker enzyme at the wavelength of 450nm, and the experiment was repeated for at least three times in each group.

2.9 Statistics

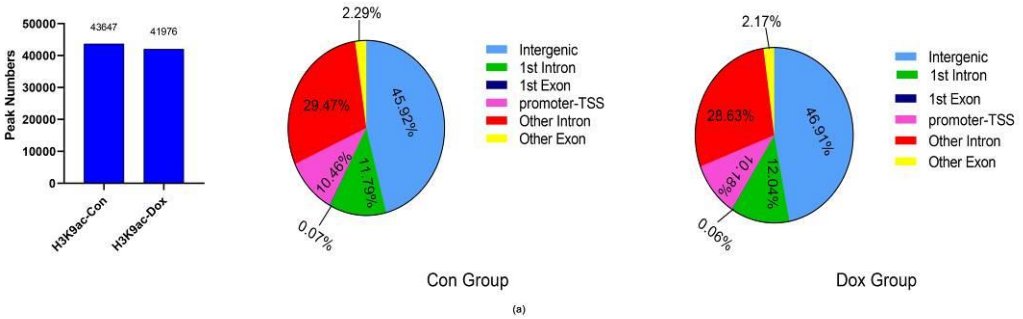
IBM SPSS Statistics 25 software and GraphPad Prism Version (Version 8) software were used for multiple sets of one-way ANOVA and two sets of unpaired T-tests, P value less than 0.05 was considered significantly.

3. Results

3.1 Doxorubicin increased the acetylation level of H3K9 in Pik3ca transcription initiation region.

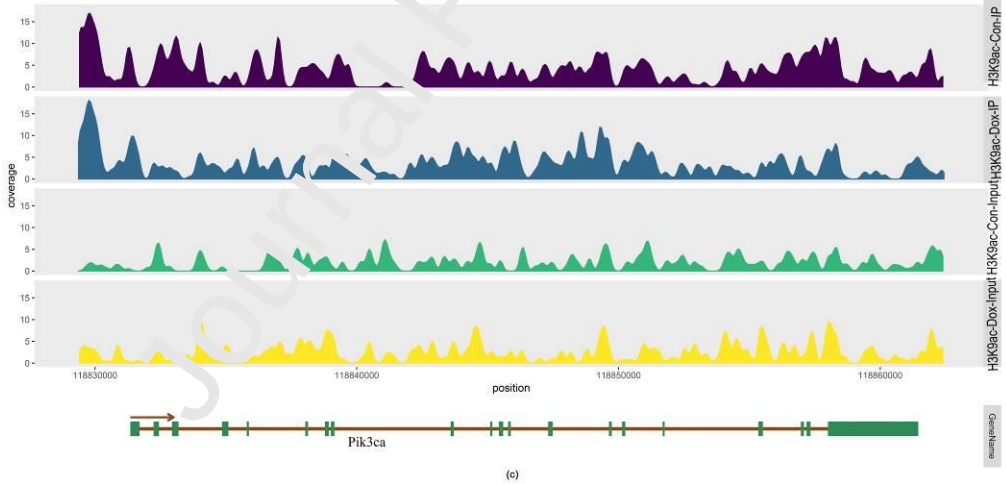
To investigate the relationship between H3K9ac and gene transcription in doxorubicin induced H9C2 cells, in this study, Chromatin immunoprecipitation (CHIP) was performed with H3K9ac antibody. Peak calling using MACS2 software (Version 2.1.1) identified that the number of short sequences of H3K9ac antibody binding in the control group was 43647, and the number of short sequences binding in the experimental group was 41976. the location of peak was analyzed, as shown in Fig.1c, in the control group,10.46% of the 1046 genes was located in promoter-TSS, while the rest was located in the 1st intron (11.79%), 1st exon (0.07%), intergenic (45.92%) etc. In the experimental group, 10.18% of the 1046 genes was located in promoter-TSS, while the rest was located in the 1st intron (12.04%), 1st exon (0.06%), intergenic (46.31%) etc. Next, by matching these sequences to gene, as shown in Fig.1b, it was found that H3K9ac antibody recognized 11654 genes in the control group and 10633 genes in the doxorubicin group. The number of differentially expressed genes identified by H3K9ac antibody in the control and doxorubicin group were clearly shown by mapping the Wain diagram. The main purpose of this study was to investigate the effect of H3K9ac on gene transcription level. Therefore, we focused on genes with different H3K9ac level in the promoter region, in which Pik3ca caught our attention.

As shown in Figure 1c, the peak value of Pik3ca in experiment group was significantly different from that in control group. The transcriptional initiation site of gene Pik3ca is located between chromosome 2 118831350 and 116661456. Focused peak analysis determined a peak with significant difference between 118826200 and 1188331799 upstream of Pik3ca transcription start site on chromosome 2 (Fig.1d). In order to prove the result of CHIP-seq, the short sequences pulled down by H3K9ac antibody was used as a template to design primers that could recognize the upstream of Pik3ca transcriptional initiation region and conduct CHIP-QPCR experiment. The results showed that the number of Pik3ca transcriptional initiation sequence enriched in the doxorubicin group was approximately twice as much as that in the control group (Fig.1e). The above consequences suggested that in doxorubicin-induced cardiomyocytes, the level of H3K9ac modification in the transcription initiation region of Pik3ca was significantly increased, which may regulate the transcription of Pik3ca.

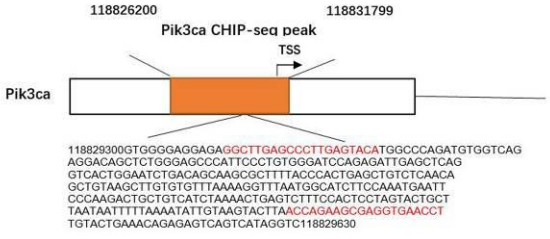


(a)

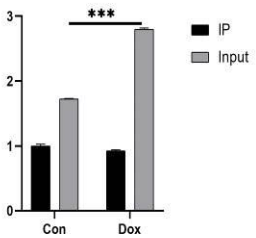
(b)



(c)



(d)



(e)

Fig.1. CHIP-seq found that the level of H3K9ac in the transcriptional initiation region of Pik3ca was increased, which promoted the transcription of Pik3ca. (a) Peak number statistics and the distribution of peak in control group and Dox group. (b) Genes number statistics and peak associated genes statistics (Wayne figure). (c) Peak differences in H3K9ac levels in the transcription initiation region of gene Pik3ca between the control group and the Dox group. (d) The sequences of primers and their positions on chromosomes. (e) Result of CHIP-QPCR for Pik3ca gene. Con: control. Dox: doxorubicin. ***: p<0.001.

3.2 Pik3ca was up-regulated in doxorubicin - induced cardiomyocytes.

Next, Q-PCR and western blot was used to detect the expression of Pik3ca in doxorubicin-induced H9C2 cells, the cells were divided into control group (Con group) and doxorubicin group (Dox group). The results showed that the mRNA level of Pik3ca in doxorubicin group was 2 times higher than that in control group (Fig.2a). Western blot was also used to detect Pik3ca protein levels, as predicted, which was increased in the doxorubicin group (Fig.2b, 2c), indicating that the increase of histone acetylation level in the transcriptional initiation region of Pik3ca promoted its expression.

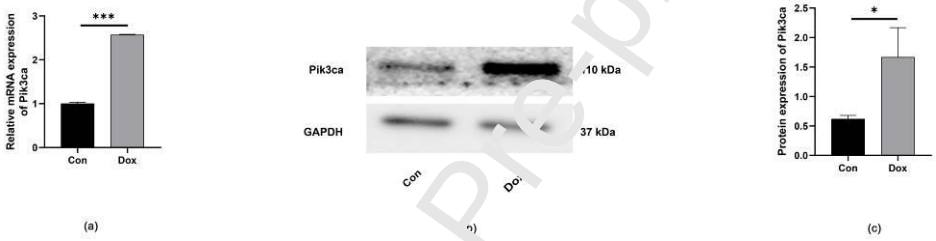
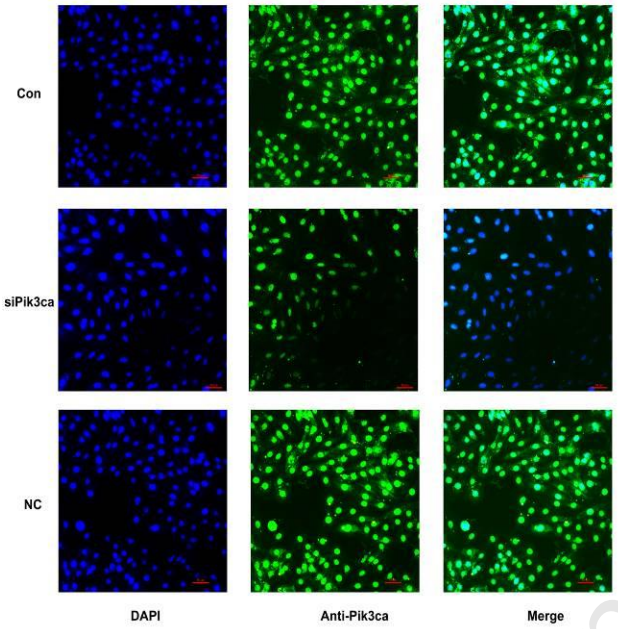


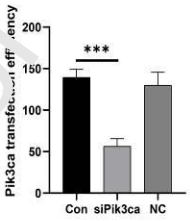
Fig.2. The expression of Pik3ca in doxorubicin induced H9C2 cardiomyocytes. H9C2 cells were cultured in complete medium for 48h, and then culture in complete medium or 1uM doxorubicin for 8h.(a) mRNA expression levels of Pik3ca and (b) protein levels of Pik3ca before and after doxorubicin intervention. (c) Statistical analysis of Pik3ca protein expression level. Con: Control. Dox: doxorubicin. ***: p<0.001, *: 0.01<p<0.05.

3.3 Pik3ca participated in apoptosis induced by doxorubicin in H9C2 cells.

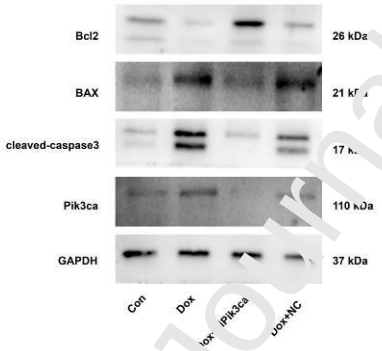
In order to prove that Pik3ca was involved in doxorubicin induced apoptosis in H9C2 cells, Pik3ca was silenced to observe doxorubicin induced apoptosis of H9C2 cells. The transfection efficiency of Pik3ca was determined by immunofluorescence assay (Fig.3a). After knocking down Pik3ca, the protein level of Pik3ca decreased significantly. Then the cells were divided into control group (Con group), doxorubicin group (Dox group), Dox+siPik3ca group and Dox + NC group. As shown in figure 3c, the transcription efficiency of Pik3ca was also measured by western blot. Comparing Dox+siPik3ca group with Dox group, protein levels of BAX and cleaved-caspase3 were down-regulated and Bcl2 was up-regulated. The results of CCK8 showed that the survival rate of H9C2 cells increased after Pik3ca knockdown (Fig.3h). Flow cytometry showed that the apoptosis rate Pik3ca knockdown decreased compared with Dox group (Fig.3i). Therefore, we demonstrated that Pik3ca promoted apoptosis of H9C2 cells. It may provide a potential therapeutic target for doxorubicin cardiomyopathy.



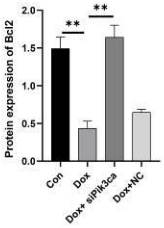
(a)



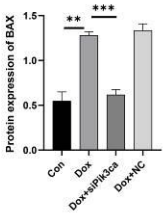
(b)



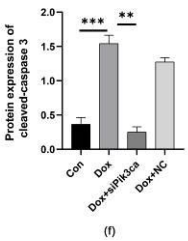
(c)



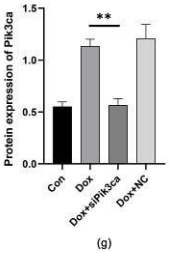
(d)



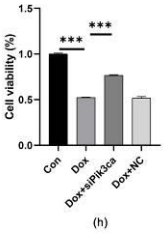
(e)



(f)



(g)



(h)

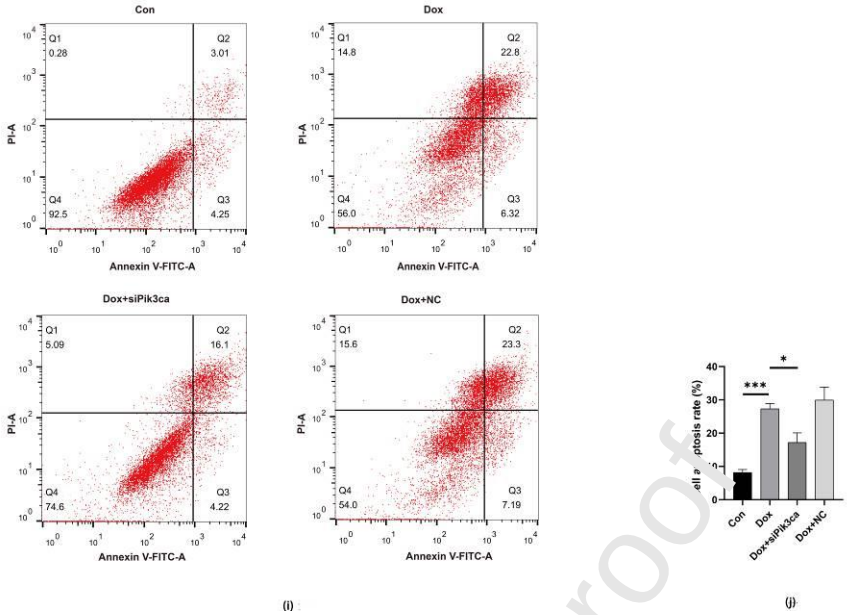


Fig.3. Knockdown Pik3ca attenuated the apoptosis of H9C2 cells induced by doxorubicin. Cultured H9C2 cells were infected with Pik3ca siRNA for 6h, followed by treatment with 1uM doxorubicin for 8h. (a) The transfection efficiency of Pik3ca was detected by immunofluorescence. (b) Immunofluorescence was used to detect transfection efficiency statistics. (c) Detection of Pik3ca proteins level and relative BAX, Bcl2, cleaved-caspase3 protein levels. (d-g) Statistical analysis of protein. (h) CCK8 assay results demonstrated that the H9C2 cells activity after Pik3ca knockdown. (i) Flow cytometry detected the apoptosis rate of H9C2 cells. (j) Statistical analysis of the flow cytometry experiment. Con: Control. Dox: doxorubicin. siPik3ca: silent Pik3ca***: P<0.001, **: <0.001P<0.01. *: 0.01<P<0.05

3.4 Doxorubicin activated the PI3K/AKT pathway in H9C2 cells.

Pik3ca is a catalytic subunit of PI3K, and the PI3K/AKT is known to be involved in the regulation of apoptosis. In this study, we tested the protein level of PI3K, AKT and P-AKT with western blot (Fig.4a). We found that protein level of PI3K, P-AKT were elevated, and the protein level of AKT barely was changed compared with control group. These results demonstrated that the PI3K/AKT signaling pathway was activated in doxorubicin induced apoptosis in H9C2 cells.

3.5 Knockdown of Pik3ca could inhibit the PI3K/AKT signaling pathway in doxorubicin induced H9C2 cells.

To verify whether Pik3ca promotes doxorubicin induced apoptosis in H9C2 cells by activating the PI3K/AKT pathway, western blot was used to detect the protein expression level of PI3K/AKT pathway after the Pik3ca was knocked down. The experiment was divided into Con group, Dox group, Dox+siPik3ca group and Dox + NC group. After silencing Pik3ca, PI3K, P-AKT decreased by two times compared with of the Dox group, and there was no significant change in the protein level of AKT (Fig.4e). All these data revealed that knockdown of Pik3ca can inhibit the PI3K/AKT signaling pathway， suggesting that Pik3ca might cause apoptosis through the PI3K/AKT pathway in doxorubicin induced H9C2 cells.

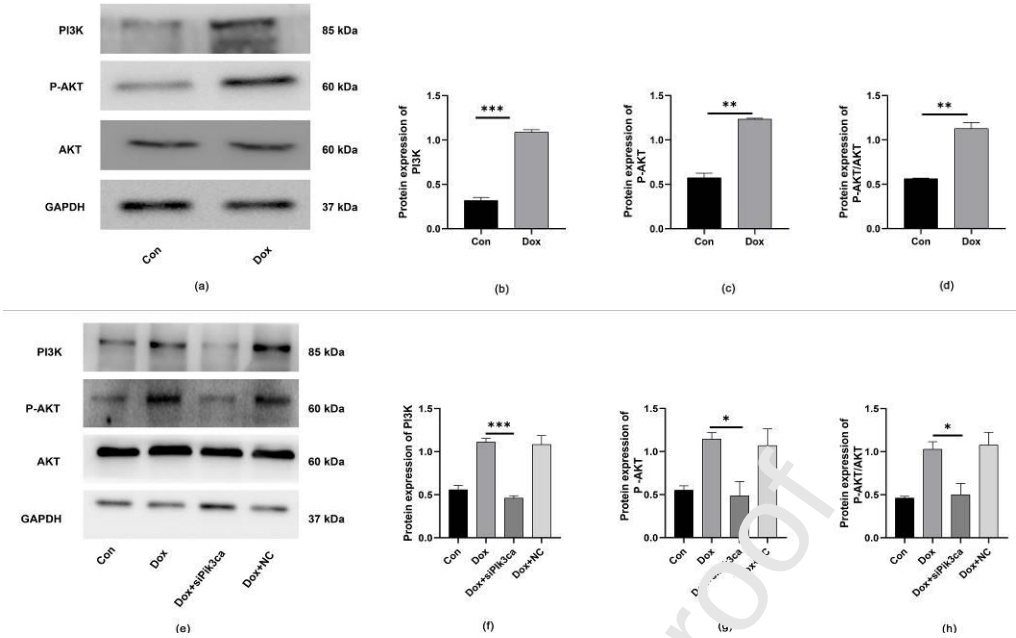


Fig.4. Pik3ca promoted apoptosis induced by doxorubicin in H9C2 cells through the PI3K/AKT pathway. H9C2 cells were cultured with Pik3ca siRNA transfection reagent for 6h, followed by doxorubicin for 8h. (a) The protein expression levels of PI3K, P-AKT, and AKT in doxorubicin induced cardiomyocytes. (b-d) Statistical analysis of PI3K, P-AKT and P-AKT/AKT protein expression levels. (e) The protein expression levels of PI3K, P-AKT and AKT were detected after silencing Pik3ca. (f-h) Statistical analysis of PI3K, P-AKT and P-AKT/AKT protein expression levels after Pik3ca knockdown. Con: control. Dox: doxorubicin. ***: P<0.001. **: 0.001<P<0.01. *: 0.01<P<0.05.

3.6 In doxorubicin induced H9C2 cells, activation of the PI3K/AKT pathway weakens the effect of silencing Pik3ca.

Finally, we used the PI3K/AKT pathway activator to conduct functional recovery experiment. In this study, the experiment was divided into Con group, Dox group, Dox+siPik3ca group and Dox+siPik3ca+740Y-P group. On the basis of silencing Pik3ca, the PI3K/AKT pathway activator 740Y-P was added to increase the protein levels of PI3K and P-AKT. Compared with silencing Pik3ca, activation of PI3K/AKT pathway promoted apoptosis of H9C2 cells. BAX and cleaved-caspase3 protein level increased, and Bcl2 protein decreased (Fig.5a). Flow cytometry showed increased apoptosis rate (Fig.5i) and CCK8 indicated decreased cell survival rate (Fig.5k). These data suggested that activation of the PI3K/AKT pathway can partially reverse the silencing effect of Pik3ca. So far, it can be said that Pik3ca promoted doxorubicin induced apoptosis in H9C2 cells by activating the PI3K/AKT pathway.

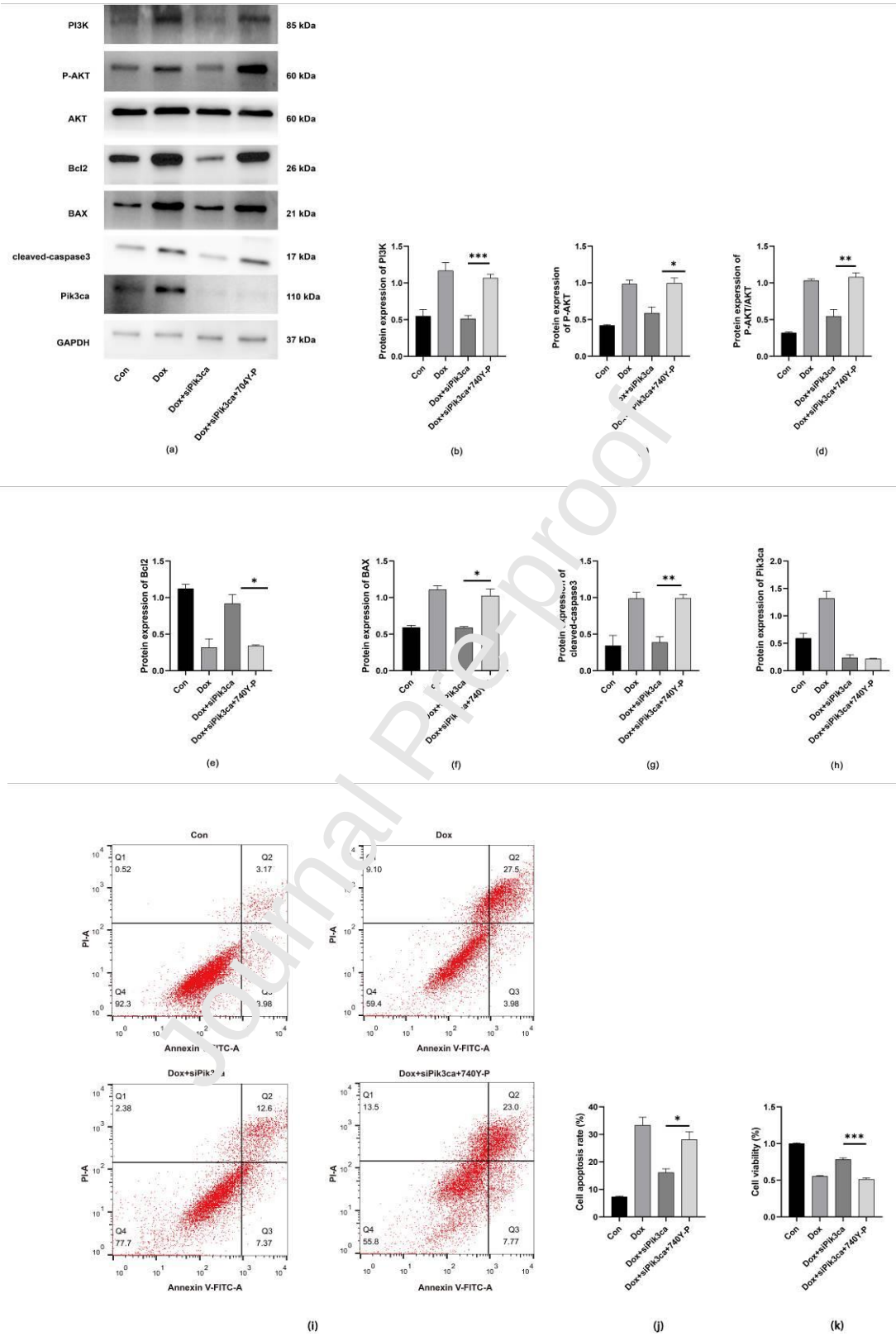


Fig 5: Activation of the PI3K/AKT pathway weakens the effect of silencing Pik3ca. H9C2 cells were cultured with Pik3ca siRNA transfection reagent for 6h, followed by doxorubicin for 8h, and finally 740Y-P for 24h. (a) the levels of PI3K, P-AKT, AKT protein and the apoptosis-relative protein BAX, Bcl2 and cleaved-caspase3 were detected after adding agonist 740Y-P. (b-h) Statistical analysis of protein expression level. (I-k) The apoptosis rate was detected by flow cytometry and the cells activity was detected by CCK8 assay (e). Con: control. Dox: doxorubicin. ***: P<0.001, **:

0.001<P<0.01. *:0.01<P<0.05.

4. Discussion

In this study, we found for the first time that doxorubicin can increase the level of H3K9ac in the transcription initiation region of Pik3ca in H9C2 cells, and for the first time confirmed the up-regulation of Pik3ca expression in doxorubicin induced H9C2 cells. Meanwhile, Pik3ca can promote doxorubicin induced H9C2 cell apoptosis through the PI3K/AKT signaling pathway. In conclusion, effective prevention and treatment of cells apoptosis was the key to the treatment of doxorubicin-induced H9C2 cells, and this study provides an important theoretical basis for apoptosis of H9C2 cells induced by doxorubicin.

In eukaryotes, histones are an octamer around which DNA is tightly packed to form chromatin nucleosomes. When the amino terminus of a histone binds to the group acetyl, the positive charge around the histone is synthesized, and histones isolated from tightly bound DNA are more likely to bind to gene promoters, enhancers, or silencing elements to regulate gene expression[15, 16]. In our study, immunoprecipitation of chromatin was performed with H3K9ac antibody. Though CHIP-seq analysis, it was found that the H3K9ac level in the transcription initiation region of some genes was significantly increased. Therefore, we hypothesized that H3K9ac may affect the transcription level of these genes. However, after QPCR detection, it was found that the transcription level of some genes did not change compared with the control group. In other words, changes in acetylation levels in the transcription initiation regions of certain genes demonstrated by CHIP alone, which do not necessarily indicate changes in the transcription level of that gene. If transcriptome sequencing is used to detect changes in gene transcription levels, combined with CHIP-seq analysis, the results may be a little better.

Pik3ca encodes the P110 catalytic subunit, PI3Kp110 α , of Class Ⅰ Phosphatidylino-sitol3-kinases(PI3Ks)[17]. Multiple studies have shown that mutations of Pik3ca in cancer can activate the PI3K/AKT pathway and promote unlimited cell proliferation[18, 19]. Although more and more evidence has shown that Pik3ca can regulate myocardial fibrosis and oxidative stress through the PI3K/AKT signaling pathway[20], the regulatory effect of Pik3ca on PI3K/AKT in doxorubicin-induced H9C2 cells was not clear. This study demonstrated for the first time that Pik3ca expression was up-regulated in doxorubicin-induced H9C2 cells and activated PI3K/AKT. Pik3ca can directly activated PI3K after binding to PI3K, rather than making PI3K bind to the growth factor receptor with phosphorylated tyrosine residue to change its phosphorylation level. Therefore, PI3K was detected in this study, rather than P-PI3K.

The PI3K/AKT signaling pathway plays an important role in the regulation of various cells function. PI3K/AKT pathway has been shown to be closely related to ROS production, oxidative stress and mitochondrial dysfunction[21], autophagy[22],etc. Previous studies have shown that activation of the PI3K/AKT pathway promoted the occurrence of cancer and it has become an important target for cancer therapy[23]. However, the activation or inhibition of the PI3K/AKT pathway in heart disease has not been determined, and its relationship with cardiomyocytes apoptosis was also uncertain[24, 25]. Glycogen synthase kinase GSK3 β is the downstream target protein of PI3K/AKT signaling pathway, which is crucial for the biological activity and function of

H9C2 cells[26]. Studies have shown that PI3K/AKT signaling pathway was inhibited in myocardial ischemia-reperfusion injury, which promoted the phosphorylation level of GSK3β, and then induced the apoptosis of myocardial cells through mitochondrial apoptosis[27]. Li Lin et al. found that cultured H9C2 cells with doxorubicin 1um for 16h, the PI3K/AKT signaling pathway promoted the expression of P-GSK3β, thereby reducing the apoptosis of cardiomyocytes[28]. In summary, the role of P-GSK3β in cardiomyopathy remained controversial. In this study, H9C2 cells were cultured with 1uM doxorubicin for 8h, and it was found that the expression of Pik3ca was significantly increased, activating the PI3K/AKT signaling pathway and promoting the apoptosis of H9C2 cells, which was consistent with previous results[29]. However, the regulatory effect of Pik3ca/PI3K/AKT on P-GSK3β in doxorubicin cardiomyopathy was still uncertain, which needed further verification.

This study provided a new therapeutic target for doxorubicin cardiomyopathy, but we only validated it at the cellular level.

5. Conclusions

In doxorubicin induced H9C2 cells, the level of H3k9ac in the transcriptional initiation area of Pik3ca was increased, which promoted the transcription of Pik3ca. Meanwhile, Pik3ca mediated doxorubicin induced apoptosis of H9C2 cells through the PI3K/AKT signaling pathway.

6.Declaration of Competing Interest

The authors declare that they have no competing interests for the publication of this article.

7.Acknowledgments

The study was supported by the Liaoning Province Central Guiding Local Science and Technology Foundation (NO.2019JH6/10400005)

References:

[1]. Renu, K., et al., Molecular mechanism of doxorubicin-induced cardiomyopathy - An update. Eur J Pharmacol, 2018. 818: p. 241-253.

[2]. Ferreira, A., et al., Altered mitochondrial epigenetics associated with subchronic doxorubicin cardiotoxicity. Toxicology, 2017. 390: p. 63-73.

[3]. Chen, Y., et al., Shengmai injection improved doxorubicin-induced cardiomyopathy by alleviating myocardial endoplasmic reticulum stress and caspase-12 dependent apoptosis. Biomed Res Int, 2015. 2015: p. 952671.

[4]. Bartlett, J.J., P.C. Trivedi and T. Pulinilkunnil, Autophagic dysregulation in doxorubicin cardiomyopathy. J Mol Cell Cardiol, 2017. 104: p. 1-8.

[5]. Wallace, K.B., V.A. Sardao and P.J. Oliveira, Mitochondrial Determinants of Doxorubicin-Induced Cardiomyopathy. Circ Res, 2020. 126(7): p. 926-941.

[6]. Kramer, A. and G.A. Challen, The epigenetic basis of hematopoietic stem cell aging. Semin Hematol, 2017. 54(1): p. 19-24.

[7]. Placek, K., J.L. Schultze and A.C. Aschenbrenner, Epigenetic reprogramming of immune cells in

injury, repair, and resolution. J Clin Invest, 2019. 129(8): p. 2994-3005.

[8]. Chu, C.H., et al., Histone acetylation is essential for ANG-II-induced IGF-IIR gene expression in H9c2 cardiomyoblast cells and pathologically hypertensive rat heart. J Cell Physiol, 2012. 227(1): p. 259-68.

[9]. Hu, J.F., et al., Allele-specific histone acetylation accompanies genomic imprinting of the insulin-like growth factor II receptor gene. Endocrinology, 2000. 141(12): p. 4428-35.

[10]. Guo, P., et al., The Histone Acetylation Modifications of Breast Cancer and their Therapeutic Implications. Pathol Oncol Res, 2018. 24(4): p. 807-813.

[11]. Jeppesen, P., Histone acetylation: a possible mechanism for the inheritance of cell memory at mitosis. Bioessays, 1997. 19(1): p. 67-74.

[12]. Pokholok, D.K., et al., Genome-wide map of nucleosome acetylation and methylation in yeast. Cell, 2005. 122(4): p. 517-27.

[13]. Li, C., et al., Hypomethylation at non-CpG/CpG sites in the promoter of HIF-1α gene combined with enhanced H3K9Ac modification contribute to maintain higher HIF-1α expression in breast cancer. Oncogenesis, 2019. 8(4): p. 26.

[14]. Zhou, W., et al., Acetylation of H3K4, H3K9, and H3K27 mediated by p300 regulates the expression of GATA4 in cardiocytes. Genes Dis, 2019. 6(3): p. 312-325.

[15]. Li, X., X. Qian and Z. Lu, Local histone acetylation by ACSS2 promotes gene transcription for lysosomal biogenesis and autophagy. Autophagy, 2017. 13(10): p. 1790-1791.

[16]. Hanf, A., et al., The anti-cancer drug doxorubicin induces substantial epigenetic changes in cultured cardiomyocytes. Chem Biol Interact, 2019. 313: p. 108834.

[17]. Kinross, K.M., et al., Ubiquitous expression of the Pik3caH1047R mutation promotes hypoglycemia, hypoinsulinemia, and organomegaly. FASEB J, 2015. 29(4): p. 1426-34.

[18]. Spangle, J.M., et al., PI3K/AKT Signaling Regulates H3K4 Methylation in Breast Cancer. Cell Rep, 2016. 15(12): p. 2692-704.

[19]. Yang, X., et al., PM (2.5)-induced ADRB2 hypermethylation contributed to cardiac dysfunction through cardiomyocytes apoptosis via PI3K/Akt pathway. Environ Int, 2019. 127: p. 601-614.

[20]. Qian, W., et al., Wogonin Attenuates Isoprenaline-Induced Myocardial Hypertrophy in Mice by Suppressing the PI3K/Akt Pathway. Front Pharmacol, 2018. 9: p. 896.

[21]. Yu, P., et al., Elabela alleviates myocardial ischemia reperfusion-induced apoptosis, fibrosis and mitochondrial dysfunction through PI3K/AKT signaling. Am J Transl Res, 2020. 12(8): p. 4467-4477.

[22]. Ba, L., et al., Allicin attenuates pathological cardiac hypertrophy by inhibiting autophagy via activation of PI3K/Akt/mTOR and MAPK/ERK/mTOR signaling pathways. Phytomedicine, 2019. 58: p. 152765.

[23]. Tewari, D., et al., Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: A novel therapeutic strategy. Semin Cancer Biol, 2019.

[24]. Chen, L., et al., Salidroside suppressing LPS-induced myocardial injury by inhibiting ROS-mediated PI3K/Akt/mTOR pathway in vitro and in vivo. J Cell Mol Med, 2017. 21(12): p. 3178-3189.

[25]. Li, H., et al., Paeonol and danshensu combination attenuates apoptosis in myocardial infarcted rats by inhibiting oxidative stress: Roles of Nrf2/HO-1 and PI3K/Akt pathway. Sci Rep, 2016. 6: p. 23693.

[26]. Sulaiman, D., et al., Paraoxonase 2 protects against acute myocardial ischemia-reperfusion injury by modulating mitochondrial function and oxidative stress via the PI3K/Akt/GSK-3β RISK pathway.

J Mol Cell Cardiol, 2019. 129: p. 154-164.
[27]. Ghaderi, S., et al., Role of glycogen synthase kinase following myocardial infarction and ischemia-reperfusion. Apoptosis, 2017. 22(7): p. 887-897.
[28]. Li, L., et al., Shenmai Injection Protects Against Doxorubicin-Induced Cardiotoxicity via Maintaining Mitochondrial Homeostasis. Front Pharmacol, 2020. 11: p. 815.
[29]. Sahu, R., et al., Wheat phenolics suppress doxorubicin-induced cardiotoxicity via inhibition of oxidative stress, MAP kinase activation, NF-kappaB pathway, PI3K/Akt/mTOR impairment, and cardiac apoptosis. Food Chem Toxicol, 2019. 125: p. 503-519.

Conflict of interest

The authors declare that we have no competing interests for the publication of this article and follow the requires of this journals that have been submitted.