Analysis of the Efficacy of Metformin Against Doxorubicin Cardiotoxicity and Exploration of its Molecular Mechanism

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Research Article

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Abstract

Doxorubicin is a very effective broad-spectrum anti-tumor drug, but it can cause dose-dependent cardiotoxicity and ultimately lead to heart failure. Previous studies have found that metformin exerts a cardioprotective effect through AMP-activated protein kinase (AMPK), but its effect on doxorubicin cardiotoxicity is still unclear. In order to investigate whether and how AMPK affects the ability of metformin to regulate the cardiotoxicity of doxorubicin, we have conducted two parts: clinical research and basic research. We found that metformin can reduce doxorubicin cardiotoxicity through clinical retrospective case-control study. Based on this, animal experiments were conducted to explore the molecular mechanism, and it was found that metformin was not associated with AMPK pathway, an important pathway of energy metabolism in the body, and this pathway did not play a protective role in doxorubicin induced cardiotoxicity. The reason may be related to decreased glucose utilization and mitochondrial autophagy of cardiomyocytes.

1. Introduction

Doxorubicin (DOX), belongs to the family of anthracycline antibiotics, its pharmacological effect is to intercalate with human DNA, thereby blocking tumor cell proliferation and inducing apoptosis^[1], it is commonly used in clinical practice as a broad-spectrum anti-tumor drug. Doxorubicin has a therapeutic effect on a variety of malignant tumors, including breast cancer, lymphoma, sarcoma and certain types of leukemia. Doxorubicin is administered through a central vein or a peripheral vein, and the administration time is a few minutes. However, its side effects, especially cardiotoxicity, severely restrict its clinical use, including various types of arrhythmia, pericardium and myocarditis, acute hypertension, congestive heart failure and even death^[2]. The mechanism of doxorubicin cardiotoxicity has always been a hot spot in tumor treatment.

Studies have found that mitochondrial damage plays a central role in doxorubicin cardiotoxicity. Targeted drugs that enhance mitochondrial quality control and promote oxidative phosphorylation metabolism are expected to be effective against doxorubicin-induced cardiac injury. Metformin (MET) is an oral hypoglycemic drug that helps control blood sugar. Many studies have found that metformin can not only lower blood sugar, but also has cardiovascular protection and anti-atherosclerosis effects. For example, the UK Prospective Diabetes Research Center (UKPDS) has followed up for 7 years in newly diagnosed type 2 diabetic populations with previous major cardiovascular events (myocardial infarction accounted for 35%). The results show that metformin has a significant cardioprotective effect. The possible mechanisms mainly include: metformin can reduce insulin resistance, reduce liver glucose output, and reduce cardiovascular risk factors^[3]; reduce body weight and improve body lipid distribution, such as total cholesterol and low-density lipoprotein levels^[4]; improve fibrinolysis and reduce the risk of thrombosis^[5]. In recent years, new evidence has shown that metformin has shown good effects in the prevention and treatment of cancer, such as research found in the course of chemotherapy, combined use of metformin can reduce the incidence of breast cancer^[6], esophageal cancer^[7], cholangiocarcinoma^[8], endometrial cancer and other cancer-related mortality. However, in the study of cardiotoxicity caused by doxorubicin only in vitro mouse cardiomyocytes^[9] and in vivo mouse heart experiments^[10] found that metformin may have an anti-doxorubicin cardiotoxic effect, but the mechanism is not yet in-depth, the results of ongoing and completed clinical studies on the cardiotoxicity of metformin against doxorubicin have not been released.

AMP-activated protein kinase (AMPK) is an enzyme involved in the energy homeostasis of the body's cells, and is expressed in metabolic organs such as the brain, liver, fat cells, and skeletal muscle. The AMPK pathway is activated in the body's stress response when adenosine triphosphate (ATP) is depleted (such as hypoglycemia, hypoxia, myocardial ischemia, heat shock, etc.), and positively regulates and supplements the cellular ATP supply pathway through fatty acid oxidation and glucose transport, and reduce ATP consumption^[11]. The activation of the AMPK pathway can maintain the body's cell energy reserve by inhibiting the ATP consumption process, and promote the biosynthesis of glycogen, triglycerides and proteins and other macromolecules. Therefore, AMPK is the switch of energy in human cells and is called the "master regulator of energy metabolism" of the human body.

Previous studies have suggested that the activation of the AMPK pathway may mediate the protective effect of metformin on doxorubicin cardiotoxicity^[12, 13], but recent studies have found that the use of AMPK activator—MK8722 (a kind of AMPK to 12 mammalian AMPK, the most effective and direct allosteric activator of the pathway) can cause cardiac hypertrophy in rhesus monkeys (shown as an increase in the QRS amplitude of the electrocardiogram)^[14]; Our preliminary experimental results of using doxorubicin to interfere with H9C2 rat cardiomyocytes also suggest, AMPK gene overexpression can lead to cardiomyocyte apoptosis indicators-activated cysteine protease protein 3 antibody (Cleaved-Casp3) and polyadenosine diphosphate polymerase antibody (Cleaved-PARP) expression has increased, while after AMPK gene was knocked out, the expression of these apoptotic proteins was decreased. It can be

seen that the role of AMPK pathway in the occurrence and development of doxorubicin cardiotoxicity has not been fully elucidated, and it is not clear whether the AMPK pathway is involved in the improvement of metformin on doxorubicin cardiotoxicity.

In order to clarify the above problems, this study intends to carry out the following three parts of the study: firstly, starting from the clinic research, analyzing whether metformin has anti-doxorubicin cardiotoxicity; secondly, AMPK gene knockout and overexpression mice are used as the research objects, respectively. Carry out serum myocardial injury index detection, heart function detection and myocardial cell apoptosis level detection; finally, preliminary exploration of related mechanisms from energy metabolism and autophagy, so as to provide a new theory for clinical prevention and treatment of doxorubicin cardiotoxicity and practical basis.

2. Material And Methods

2.1 Clinical research

2.1.1 Research object

Inclusion criteria: in January 2016 to January 2019 in the second affiliated hospital of Xi'an JiaoTong university myeloid leukemia, non-hodgkin's lymphoma, breast cancer patients with doxorubicin periodically chemotherapy 123 study, in which 43 cases with metformin (200 mg/day, test group) and 80 cases of combination with metformin (control group). Exclusion criteria: metformin in patients with type 2 diabetes, combined use of antiarrhythmic drugs or β -blockers.

2.1.2 Research methods

Baseline data were first compared between the two groups, then self and intergroup controls were used. The changes of myocardial injury indexes [serum phosphocreatine kinase isoenzyme (CK-MB) level, lactate dehydrogenase (LDH) level] and heart failure indexes [serum B-type natriuretic peptide (BNP) level, left ventricular ejection fraction (EF), left ventricular short axis shortening rate (FS)] were retrospectively analyzed before and after medication in 2 groups. Excel was used to collect data and SPSS 18.0 software was used for statistical analysis. The above indexes of myocardial injury and heart failure were expressed as "mean $\pm$ standard deviation", and the data followed normal distribution. T-test was used for intra-group comparison and inter-group pair-in comparison, and Graphpad Prism 9.0 software was used to plot. The test level $\alpha=0.05$, when the *P* value is less than 0.05, the results are statistically different. All operations have been approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. In the attachment.

2.2 Basic research

2.2.1 AMPK gene knockout in animals

1) Research object

Twenty-four 6-week-old AMPK $\alpha 2$ knockout (AKO) mice were used as subjects and twenty-four 6-week-old wild-type C57BL/6 mice (WT) were used as control. Both groups were purchased from Jackson Laboratory, USA. During the experiment, the animals were kept in the animal house of the Second Affiliated Hospital of Xi'an Jiaotong University. The experimental plan was approved by the Laboratory Animal Management Committee of Second Affiliated Hospital of Xi'an Jiaotong University. In the attachment, and relevant measures were taken to avoid the pain of the animals during the operation. Animal room setting standards are: indoor humidity $55\pm 5^\circ\text{C}$, temperature $22\pm 2^\circ\text{C}$ and alternating light and dark 12h each. The cages are transparent, with no more than 5 mice per cage, and the mice can freely obtain food and water.

2) Research methods

AKO group and WT group were randomly divided into 4 groups, including control group (Con), metformin group (MET), doxorubicin group (DOX) and metformin combined doxorubicin group (MET + DOX), with 6 mice in each group. Metformin intervention methods: Mice in MET group and MET+DOX group were orally treated with metformin (1,1-Dimethylbiguanide hydrochloride SIGMA D5035) at 200 mg/kg/d daily from day 1 to day 6 of the experiment, while mice in Con group and DOX group were not treated with oral drug intervention and were free to obtain water and food. Doxorubicin intervention method: DOX group and MET+DOX group were intraperitoneally injected with 20mg/kg doxorubicin (Doxorubicin Hydrochloride RPI D43000-0.01) on the third day of the experiment, Con group and MET group were intraperitoneally injected with the same amount of normal saline. Mice in each group were

anesthetized on the 6th day of the experiment and underwent cardiac color doppler examination. Mice were sacrificed on the 7th day of the experiment, and blood samples were collected from abdominal aorta. Serum and heart tissue were collected as required.

1. Western Blot Analysis

The membranes were incubated with primary and secondary antibodies, and processed for chemiluminescent detection using Lumigen ECL Ultra (TMA-6 Lumigen, Southfield, MI, United States). The images were acquired by using Amersham Imager 600 and quantified with ImageJ. The antibodies against AMPK $\alpha 2$ (SC19131), PDH(SC3205), P-PDH(SC37115) and β -actin(SC3700S). Secondary antibodies (sc2004, sc-2005, sc-2020, and sc-2438) were obtained from Santa Cruz Biotechnology.

b) Cardiac color ultrasound detection

Vevo2100 cardiac ultrasonic (GE, United States) diagnostic instrument was used to examine the heart of mice one day before death. The mice were anesthetized by inhaling 2% isoflurane, and the mice were fixed on the heating platform. The hair removal cream was used to remove chest hair, the hair was wiped off with absorbent paper, and the skin was cleaned with water-soaked cotton. The coupling agent was extruded to cover the chest area. Left ventricular M-mode echocardiography was obtained on the long axis section of left ventricle beside the sternum, and the FS value of left ventricle was recorded.

c) Extraction of serum and myocardial tissue

On the 7th day of the experiment, the mice were sacrificed, and the mice were anesthetized by 2% isoflurane inhalation for 5 ~ 7 minutes using pre-sterilized surgical instruments. The mice were fixed in supine position on the workbench, the abdominal cavity was opened, and the abdominal aorta was exposed. The abdominal aorta was separated with sterilized scissors forceps. After the blood was collected from the abdominal aorta, the blood was left standing for 30min, followed by centrifugation at 3000rpm for 15min, and the serum samples were placed in the refrigerator (-80°C). The chest cavity was then opened and the heart was separated. The heart was quickly transferred to a petri dish on a cooling plate. After the heart was removed, the left ventricle was separated and rinsed with normal saline. The other part was fixed in 10% formalin solution for 24h and embedded in conventional paraffin for morphology and other experiments.

d) Serum lactate dehydrogenase (LDH) and cardiac troponin I (cTnI) were detected

Serum LDH levels were determined using an LDH ELISA kit (CST, United States). Take out the serum samples stored in the refrigerator (-80°C), first add the mouse serum samples and LDH standard to the 96-well plate, add LDH determination buffer, add 48uL to each well, add 50uL mixture (mixture = 48uL LDH determination buffer + 2uL serum sample) to each well, 3 minutes after counting, Then scan at 450nm, read the wavelength, and calculate the LDH level according to the formula required by the kit. Serum cTnI levels were determined using a cTnI ELISA kit (CST, United States). Take out the serum samples stored in the refrigerator (-80°C), add 200uL mouse serum samples and 200uL standard mouse serum samples respectively with 96-well plates, place the plates on the shaker (150rpm, 25°C) for 2 hours, and cover them with elastic plastic wrap; Then rinse for the first 5 times, add 100uL thinner to each well and 100uL HRP enzyme conjugate to each well. Then place the plate on the shaker (150rpm, 25°C) for 1 hour and cover it with elastic plastic wrap. Rinse for the second time 5 times, add 100uL TMB solution to each well, cover with elastic plastic wrap, place on a shaking table (150rpm, 25°C) for 20 minutes, add 100uL stop solution to each well, shake the mixture gently, when all the solutions change from blue to yellow, read the wavelength of the 96-well plate at 450nm, The cTnI level was calculated according to the formula required by the kit.

e) Apoptosis level of myocardial cells was detected by TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Abcom Cat.12156792910)

☒ Fixation and permeation: a) Tissue sections were fixed with fixative solution at 15-25°C for 20 minutes; b) Soak in PBS solution for 30 minutes; c) Incubate the slides in Triton X-100 working solution on ice for 2-5 minutes; d) The sections were incubated at room temperature for 1 hour.

☒ To prepare the TUNEL reaction mixture: a) Take out 100uL label solution (sample bottle 2) for 2 negative controls; b) Add total volume (50uL) of enzyme solution (sample bottle 1) to the remaining 450uL labeled solution (sample bottle 2) = total volume of 500uL; c) Stir well;

▯ The specific experimental steps are as follows: a) Carefully suck out the osmotic solution; b) Tissue sections were flushed with PBS twice; c) Wipe off excess solution to dry slices; d) Drop 50uL Tunel reaction mixture onto the slide slice; e) Cover the sections with sealing film; f) Incubate the slides in darkness at 37°C in a humid environment for 1 hour; g) Wash slides with PBS for 3 times; h) Wipe off excess solution to dry slices; i) Fluorescence microscopy/confocal analysis.

2.2.2 AMPK gene overexpression in animals

1) Research object

Nineteen six-week-old wild-type C57BL/6 mice (purchased from Jackson Laboratory, USA) were used as the research subjects.

2) Research methods

The above-mentioned mice were randomly divided into 4 groups: control group (Con group, n = 4), MK8722 group (MK group, n = 4), doxorubicin group (DOX group, n = 6), and MK8722 group combined with doxorubicin Group (MK+DOX group, n = 5). MK8722 intervention method: mice in the MK group and MK+DOX group were given oral MK8722 at 30 mg/kg/d on day 1 to 6 of the experiment. The Con group and DOX group did not receive oral drug intervention and had free access to water and food; Doxorubicin Intervention method: Doxorubicin was injected intraperitoneally at 20 mg/kg in DOX group and MK+DOX group on the second day of experiment, and the same amount of normal saline was injected intraperitoneally in Con group and MK8722 group. On the 6th day, all mice were subjected to cardiac color Doppler sonography. On the 7th day, the animals were sacrificed and the serum and myocardial tissue were collected.

2.2.3 Molecular mechanism of AMPK in doxorubicin-induced cardiotoxicity

1) Research object

Seven 6-week-old wild-type C57BL/6 mice (Wild Type, WT) and eight 6-week-old AMPKα2 knockout (AKO) mice were used as experimental subjects, which were purchased from the Jackson Laboratory in the United States. The feeding method is the same as before.

2) Research methods

The WT group was randomly divided into a control group (WT Con, n = 3) and a pepA / E64d (Thermo, the United States) intervention group (WT + pepA / E64d, n = 4), and the AKO group was also randomly divided into a control group (AKO Con, n = 4) And pepA / E64d intervention group (AKO+ pepA / E64d, n = 4). Since autophagy is a dynamic process, in order to fully describe the dynamic change process of autophagy, we artificially use aspartic protease inhibitor (pep A) and cysteine protease inhibitor (E64d) to block the process of autophagy, Inhibit the degradation of LC3B-▯, and then completely describe the dynamic change process of autophagy. Mice in WT+pepA/E64d group and AKO+pepA/E64d group were injected intraperitoneally with pepA at 1.5mg/kg and E64-d at 1.5mg/kg 4 hours before sacrifice. Western blot protein quantitative method was used to determine the expression levels of LC3B-▯ protein in the total components of cardiomyocytes, mitochondria, and cytoplasm.

Statistical analysis

Mean ± standard deviation (SD) is used to represent the overall distribution of data, t-test is used to compare data between two groups, and one-way analysis of variance is used to compare data between multiple groups. GraphPad Prism 9.0 and SPSS 18.0 software were used for data analysis and mapping. The test level α=0.05, when the P value is less than 0.05, the results are statistically different.

3. Results

3.1 Clinical research results

3.1.1 Comparison of baseline data between the two groups

A total of 43 patients in the experimental group and 80 patients in the control group were included in this study. The baseline data of the two groups are shown in **Table 1**. As can be seen from the table, there was no statistical difference in baseline data between the

two groups ($P>0.05$).

Table 1 Comparison of baseline data between the two groups

Items	Control groupN=80	Test groupN=43	P value
Gender (Male/female)	47/43	24/19	>0.05
AgeX±S	60.59±11.81	61.90±8.98	>0.05
Underlying heart disease%	7/808.75%	4/439.30%	>0.05
CK-MBU/L	15.57±7.58	16.85±9.49	>0.05
LDHU/L	200.77±98.50	230.42±93.61	>0.05
BNPpg/ml	193.58±102.80	195.06±74.84	>0.05
EF%	62.02±6.04	60.00±8.01	>0.05
FS%	36.28±5.53	35.83±4.06	>0.05

3.1.2 Comparison of the changes of each index between the two groups before and after chemotherapy

Changes of myocardial indexes before and after chemotherapy in 80 patients in control group and 43 patients in test group were shown in **Table 2** and **Figure 1**. The levels of CK-MB, LDH and BNP in the control group after chemotherapy were significantly higher than those before chemotherapy, while EF and FS in the control group were significantly lower than those before chemotherapy ($P<0.05$); The levels of CK-MB, LDH and BNP in test group after chemotherapy were significantly lower than those before chemotherapy ($P<0.05$), EF and FS had no significant change compared with before chemotherapy ($P>0.05$). After chemotherapy, the levels of CK-MB, LDH and BNP in the test group were significantly lower than those in the control group, while EF and FS were significantly higher than those in the control group ($P<0.05$).

Table 2 Comparison of indicators between the two groups before and after chemotherapyX±S

	Control group		Test group	
	Con-Pre	Con-Post	Met-Pre	Met-Post
CK-MBU/L	15.57±7.58	23.75±9.43 [#]	16.85±9.49	11.02±7.11 ^{*△}
LDHU/L	200.77±98.50	400.25±268.29 [#]	230.42±93.61	190.6±109.04 ^{*△}
BNPpg/ml	193.58±102.80	348.43±147.34 [#]	195.06±74.84	87.61±57.24 ^{*△}
EF%	62.02±6.04	46.05±7.03 [#]	60.00±8.01	65.51±4.03 ^{*△}
FS%	36.28±5.53	30.38±4.97 [#]	35.83±4.06	38.47±3.65 ^{*△}

3.2 Basic research results

3.2.1 AMPK gene knockout results

1)Inhibition of AMPK pathway in myocardium

As shown in **Figure 2**, the expression level of AMPKα2 protein in myocardium of mice in AKO group was significantly lower than that in WT group. Quantitative analysis showed that the inhibition rate of AMPKα2 in AKO group was 50% compared with WT group, which met the inhibition requirements and could be used for subsequent experimental studies.

2) Results of heart function test in mice

The results of left ventricular FS in each group are shown in **Table 3** and **Figure 3**. Compared with the control group, metformin single drug intervention had no significant effect on FS in both groups (WT group: 37.1±2.4 vs 38.5±6.2, AKO group: 38.3±1.5 vs 39.5±4.2, $P>0.05$); Adriamycin single drug intervention significantly reduced FS in both groups (WT group: 29.2±1.8 vs 38.5±6.2, AKO group:

33.6±2.0 vs 39.5±4.2, $P<0.05$), but the degree of decrease in AKO group was less than that in WT group (33.6±2.0 vs 29.2±1.8, $P<0.05$). Metformin combined with doxorubicin could restore the reduced FS of WT mice (36.3±4.81 vs 29.2±1.8, $P<0.05$), but there was no significant improvement in AKO group's reduced FS (34.3±2.7 vs 33.6±2.0, $P>0.05$).

Table 3 Results of FS in WT group and AKO group X±S

FS%	WT				AKO			
	Con	MET	DOX	MET+DOX	Con	MET	DOX	MET+DOX
	38.5±6.2	37.1±2.4	29.2±1.8*	36.3±4.81△	39.5±4.2	38.3±1.5	33.6±2.0#	34.3±2.7

3) Results of myocardial injury index in serum of mice

The results of serum LDH and cTnl in each group were shown in **Table 4** and **Figure 4**. Compared with the control group, metformin monotherapy had no significant effect on serum LDH and cTnl in WT group and AKO group (WT group: LDH: 142.4±42.6 vs 155.7±113.6, cTnl: 15.2±3.1 vs 13.6±8.4; AKO group: LDH: 165.2±105.0 vs 147.6±107.9, cTnl: 11.6±4.0 vs 11.1±9.6, $P>0.05$), doxorubicin monotherapy significantly increased serum LDH and cTnl in both groups (WT group: LDH: 335.2±108.8 vs 155.7±113.6, cTnl: 20.6±9.6 vs 13.6±8.4; AKO group: LDH: 198.0±2.0 vs 147.6±107.9, cTnl: 14.7±5.7 vs 11.1±9.6, $P<0.05$), but the level of serum LDH and cTnl in AKO group was less than that in WT group (LDH: 198.0±2.0 vs 335.2±108.8, cTnl: 14.7±5.7 vs 20.6±9.6, both $P<0.05$). Compared with doxorubicin monotherapy group, metformin combined with doxorubicin could reduce serum elevated LDH and cTnl in WT group (LDH: 207.5±20.7 vs 335.2±108.8, cTnl: 10.6±9.0 vs 20.6±9.6, $P<0.05$), but there was no significant improvement in elevated LDH and cTnl in AKO group (LDH: 200.9±151.1 vs 198.0±2.0, cTnl: 11.1±7.6 vs 14.7±5.7, $P>0.05$).

Table 4 Results of serum myocardial injury index in WT group and AKO group X±S

	WT				AKO			
	Con	MET	DOX	MET+DOX	Con	MET	DOX	MET+DOX
LDH	155.7±113.6	142.4±42.6	335.2±108.8*	207.5±20.7△	147.6±107.9	165.2±105.0	198.0±2.0#	200.9±151.1
cTnl	13.6±8.4	15.2±3.1	20.6±9.6*	10.6±9.0△	11.1±9.6	11.6±4.0	14.7±5.7#	11.1±7.6

4) Results of myocardial apoptosis level detection

As shown in **Figure 5**, compared with the control group, metformin alone had no significant effect on the apoptosis level of myocardial cells in the two groups ($P>0.05$), doxorubicin alone significantly increased the apoptosis level of myocardial cells in both groups ($P<0.01$), but the increased degree of myocardial apoptosis in AKO group was less than that in WT group ($P<0.05$). Compared with doxorubicin alone, metformin combined with doxorubicin improved the apoptosis level of WT group ($P<0.05$), but did not significantly improve the apoptosis level of AKO group ($P>0.05$).

3.2.2 AMPK overexpression experiment results

1)Verification of overexpression effect of myocardial AMPK pathway

It can be seen from **Figure 6** that compared with the control group, the expression level of P-AMPK/AMPKα2 protein in the myocardium of the mice in the MK group and the MK+DOX group was significantly increased; Compared with Con group, the protein expression of p-AMPK /AMPKα2 in myocardium of MK group and MK+DOX group was increased by 36% and 13%, respectively, meeting the overexpression requirements of AMPKα2 pathway, which can be used for subsequent experimental studies.

2) Results of heart function test in mice

The detection results of left ventricular EF and FS of each group of mice are shown in **Table 5** and **Figure 7**. Compared with the control group, the intervention of MK8722 alone had no effect on the EF and FS of mice (EF: 67.7±8.6 vs 75.1±8.7, FS: 37.1±7.9 vs 43.5±7.4, $P>0.05$); doxorubicin single-agent intervention It can reduce both EF and FS of mice (EF: 63.1±4.5 vs 75.1±8.7, FS:33.5±3.1 vs 43.5±7.4, both $P<0.05$). Compared with the DOX group, the co-intervention of MK8722 and doxorubicin could reduce the EF and FS of mice more significantly (EF: 42.0±6.7 vs 63.1±4.5, FS: 20.2±3.9 vs 33.5±3.1, both $P<0.05$).

Table 5 EF and FS test results in each group

	Con	MK	DOX	MK+DOX
EF	75.1±8.7	67.7±8.6	63.1±4.5*	42.0±6.7**
FS	43.5±7.4	37.1±7.9	33.5±3.1*	20.2±3.9**

3) Results of myocardial injury index in serum of mice

The serum LDH and cTnl of each group of mice are shown in **Table 6** and **Figure 8**. Compared with the control group, MK8722 single-drug intervention had no effect on mouse serum LDH and cTnl (LDH: 22.4±8.5 vs 30.6±9.7, cTnl: 6.9±1.8 vs 4.5±1.1, $P>0.05$); doxorubicin alone intervention can increase serum LDH and cTnl in mice (LDH: 89.5±36.8 vs 30.6±9.7, cTnl: 13.4±5.07 vs 4.5±1.1, $P<0.05$). Compared with the Dox group, the joint intervention of MK8722 and doxorubicin can increase the LDH and cTnl of mice more significantly (LDH: 121.7±70.4 vs 89.5±36.8, cTnl: 25.4±15.7 vs 13.4±5.07, $P<0.05$).

Table 6 Detection results of serum myocardial injury indexes in mice

	Con	MK	DOX	MK+DOX
LDH	30.6±9.7	22.4±8.5	89.5±36.8*	121.7±70.4**
cTnl	4.5±1.1	6.9±1.8	13.4±5.07*	25.4±15.7**

3.2.3 Molecular mechanism of AMPK in doxorubicin-induced cardiotoxicity

1) Effects of APMK gene knockout and overexpression on the expression of PDH and P-PDH in mouse myocardium

The AMPK gene knockout experiment found that compared with the WT group (**Figure 9**), there was no difference in the expression of PDH protein in the myocardium of mice in the AKO group, but the expression level of P-PDH protein was significantly reduced ($P<0.05$). The AMPK gene overexpression (MK8722 intervention) experiment found (**Figure 10**) that compared with the Con group, there was no difference in the expression of PDH protein in the myocardium of mice in the MK group and the MK+DOX group, but P-PDH in the MK group and the MK+DOX group the protein expression level was significantly increased ($P<0.05$).

2) Effect of AMPK knockout on the expression of autophagy-related protein LC3B in cardiomyocytes

Compared with the WT pepA/E64d group, the expression of LC3B protein in the total component and mitochondrial component of cardiomyocytes in the AKO pepA/E64d group was significantly increased (**Figure 11A, B**, $P<0.01$), but in the cytoplasm The expression in the components was significantly reduced (**Figure 11C**, $P<0.01$).

Discussion

1. The protective effect of metformin on doxorubicin-mediated cardiotoxicity

The results of the clinical retrospective analysis of this study found that the blood CK-MB, LDH, and BNP levels of myocardial injury indicators in the control group without metformin after chemotherapy increased compared with those before chemotherapy, and the levels of cardiac function EF and FS decreased compared with those before chemotherapy. The blood CK-MB, LDH and BNP levels in the test group combined with metformin were significantly lower after chemotherapy than before chemotherapy; comparing the above indicators after chemotherapy in the two groups of patients, it was found that the blood CK-MB, LDH and BNP levels in the test group after chemotherapy were higher than those in the control group, and the EF and FS value increased compared with the control group, indicating that the combined application of metformin has a certain preventive effect on doxorubicin cardiotoxicity.

The cardiotoxicity of doxorubicin is related to cell metabolism, and mitochondrial damage plays a central role in this process^[15]. Doxorubicin enters myocardial mitochondria, which can accept electrons from complex 1 to form free radicals. Subsequent delivery of an electron to oxygen produces superoxide, which leads to mitochondrial damage and cellular oxidative stress. Mitochondrial dysfunction after doxorubicin treatment can lead to the preferential use of fatty acid β -oxidation for energy metabolism to anaerobic glycolysis^[16], maintaining mitochondrial integrity and mitochondrial oxidative metabolism has a protective effect on doxorubicin cardiotoxicity^[17]. In vitro cell experiments suggest that metformin can counteract the mitochondrial DNA damage of cardiomyocytes

caused by doxorubicin^[18]. Therefore, metabolic targeted drugs that enhance mitochondrial quality control and promote oxidative phosphorylation, such as the metformin used in this study, are expected to combat doxorubicin-induced cardiac damage. At present, a number of clinical trials combining metformin into chemotherapy regimens have been carried out. The follow-up results need to be further tracked.

2. The beneficial actions of Metformin may be AMPK independent in the heart (at least some part).

Combined with reports in the literature, the myocardial protective effect of metformin may be related to cell energy metabolism. Therefore, we selected AMPK pathways that are closely related to energy metabolism to explore the mechanism. In experimental studies, the expression of α subunit is mainly used as the total AMPK and AMPK protein activity. According to the evidence, the myocardial tissue is mainly $\alpha 2$ subunit, and we mainly detected AMPK $\alpha 2$ protein for follow-up experimental research. First, the AMPK $\alpha 2$ pathway knockout (AKO) mouse experiment was carried out, and it was found that the single-agent intervention of doxorubicin reduced FS in both groups, and increased LDH, cTnI and cardiomyocyte apoptosis, but the degree of FS decreased in the AKO group lighter than the control group, LDH, cTnI and myocardial cell apoptosis levels increased lightly than the control group; the joint intervention of metformin and doxorubicin can restore the lowered FS of the control group mice, and the increased LDH, cTnI and the apoptosis level of cardiomyocytes was improved, but there was no significant change in the above indicators in the AKO group. On the one hand, this result indicates that after metformin intervention, the cardiac function of doxorubicin-induced cardiotoxicity in mice can be improved, and myocardial damage and myocardial cell apoptosis can be reduced. On the other hand, it was suggested that the above indicators were not significantly improved after the intervention of metformin in the AKO group, that is, the mechanism of metformin's prevention and treatment of doxorubicin cardiotoxicity has nothing to do with AMPK pathway. This is consistent with the results of our previous H9C2 rat cardiomyocyte pre-experiment, that is, the simultaneous intervention of metformin and doxorubicin can make the control group's cardiomyocyte apoptosis indicators-activated cysteine protease protein 3 antibody (Cleaved-Casp3) and the expression of polyadenosine diphosphate polymerase antibody (Cleaved-PARP) was weakened, but there was no significant change in the above indicators in the AMPK $\alpha 2$ knockout cell group. Although studies have found that metformin can activate the AMPK pathway to regulate cellular oxidative stress, reduce mitochondrial dysfunction, and exert a protective effect on H9C2 cardiomyocytes induced by doxorubicin^[19]. Our results suggest that the protective effect of metformin on doxorubicin cardiotoxicity is independent of AMPK pathway.

In order to further confirm the role of AMPK pathway in doxorubicin cardiotoxicity, we carried out AMPK $\alpha 2$ overexpression animal experiments. The results found that compared with the control group, doxorubicin monotherapy can significantly reduce the EF and FS of mice. Both LDH and cTnI were significantly increased; compared with the doxorubicin single-agent intervention group, the joint intervention of MK8722 (AMPK allosteric activator) and doxorubicin can significantly reduce the EF and FS of mice, and increase the blood LDH and cTnI more significantly. obvious. The above results suggest that AMPK pathway is involved in the occurrence of doxorubicin cardiotoxicity.

3. Causes of activating AMPK pathway aggravating doxorubicin cardiotoxicity

The survival and activity of organisms depend on energy metabolism, and the balance of energy supply and demand depends on the regulation of energy metabolism. Pyruvate plays a key role in the important energy metabolism pathway of organisms-tricarboxylic acid cycle. The oxidation of acetyl-CoA derived from it will generate NADH and FADH₂, which are re-oxidized in the mitochondrial oxidative phosphorylation pathway. All cells (except mature red blood cells) are produced to maintain homeostasis of ATP. PDH acts as a link between glycolysis and citric acid cycle. P-PDH inhibits its activity by phosphorylating PDH, and P-PDH represents a decrease in glucose utilization. Studies have found that AMPK crucially maintains the activity of the rate-limiting enzyme PDH involved in the tricarboxylic acid cycle, thereby facilitating the metabolism of pyruvate to the tricarboxylic acid cycle, rather than converting it into lactic acid. AMPK co-localizes with the catalytic subunit of PDH in the mitochondrial matrix, and directly triggers the phosphorylation of PDH at serine 295 (Ser295) and serine 314 (Ser314)^[20]. Therefore, AMPK, PDH and P-PDH are closely related and have important significance in the treatment of many metabolic diseases, such as obesity, diabetes, cancer, and Alzheimer's disease^[21]. We found that in the AMPK $\alpha 2$ gene knockout experiment, there was no difference in the expression of PDH protein in the myocardium of mice in the AKO group, but the expression of P-PDH protein decreased, suggesting that glucose utilization increased after the AMPK pathway was inhibited; AMPK gene overexpression (MK8722 intervention) experiment found There was no difference in the expression of PDH protein in the myocardium of mice in the MK group and MK+DOX group, but the expression of P-PDH protein in the MK group and MK+DOX group increased, indicating that the glucose utilization rate decreased after the AMPK pathway was overexpressed.

Therefore, the cause of the adverse effect of AMPK in doxorubicin cardiotoxicity may be related to the decreased glucose utilization rate of cardiomyocytes.

AMPK acts as an energy sensor in cells, playing a key role in catabolic upregulation and anabolic inactivation. Activated AMPK regulates a variety of metabolic processes, including autophagy. MTOR activity, a downstream product of AMPK, was inhibited and autophagy was enhanced, which in turn could negatively regulate mTOR activity after autophagy enhancement^[22]. In order to further clarify the role of AMPK in the regulation of autophagy, we injected pepA and E64d into mice intraperitoneally to block the process of autophagy and inhibit the degradation of LC3B- β , and use the autophagosome marker-LC3B- β protein for autophagy. Phage detection. The results showed that the expression of LC3B- β protein in the total components of cardiomyocytes and mitochondria of AMPK α 2 knockout mice was significantly increased, but the expression in the cytoplasmic components was significantly decreased. The reason for the increase in total autophagy in the cardiomyocytes of AMPK α 2 knockout mice was analyzed because of the increase in mitochondrial autophagy, while the cytoplasmic autophagy was reduced. Therefore, the cause of the adverse effect of AMPK in doxorubicin cardiotoxicity may be related to the reduction of mitochondrial autophagy, but whether it is involved in the common mitochondrial autophagy pathway is still unclear, and further exploration is needed.

4. The shortcomings of this research

Due to the small number of patients included in the study, it is impossible to conduct a stratified study of patients, so it is necessary to continue to collect clinical data and strengthen clinical research. In the future, we will further study the molecular pathways of cardioprotection when metformin is used in combination with doxorubicin, as well as the use of metformin and the development of AMPK inhibitors for phase II clinical trials against doxorubicin cardiotoxicity. In addition, we will further confirm whether changes in glucose metabolism and/or autophagy are the cause of cardiotoxicity caused by doxorubicin.

Conclusion

In summary, our study has found that doxorubicin has cardiotoxicity in clinical application, and metformin combined with doxorubicin has a certain prevention and treatment effect on cardiotoxicity. The prevention and treatment mechanism of metformin against doxorubicin cardiotoxicity is unrelated to AMPK pathway. And AMPK pathway is involved in doxorubicin cardiotoxicity, and its mechanism may be related to decreased glucose utilization of cardiomyocytes and mitochondrial autophagy.

Declarations

Ethics approval and consent to participate: The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of the Second Affiliated Hospital of Xi 'an Jiaotong University. Written informed consent was obtained from individual or guardian participants. (The supporting documents are attached).

Consent for publication All data generated or analysed during this study are included in this published article.

Availability of data and materials All data, models, and code generated or used during the study appear in the submitted article.

Competing interests The authors declare that they have no competing interests

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Authors' contributions Jia Wei carried out experimental operations, performed data analyses and drafted the manuscript. Jin Wei is writing-reviewing and editing, responsible for the overall content of the work as guarantors. All authors read and approved the final manuscript.

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Figures

Figure 1

Comparison of indicators between the two groups before and after chemotherapy X±S

Figure 2

AMPKα2 expression in WT and AKO mice

Figure 3

Results of FS in WT group and AKO group

Notice: * Compared with the WT Con, $P < 0.01$; Δ Compared with the WT DOX, $P < 0.05$; # Compared with the WT DOX, $P < 0.05$. Data are expressed as mean $\pm$ SEM and analyzed by using unpaired student t-test.

Figure 4

Results of serum myocardial injury index in WT group and AKO group

Notice: *Compared with the WT Con, $P < 0.01$; ΔCompared with the WT DOX, $P < 0.05$; # Compared with the WT DOX, $P < 0.05$. Data are expressed as mean $\pm$ SEM and analyzed by using unpaired student t-test.

(a) TUNEL staining results of WT group

(b) TUNEL staining results of AKO group

(c) Changes in apoptosis ratio of myocardial cells

Figure 5

Evaluation of myocardial cell apoptosis in WT and AKO groups

Notice: TUNEL positive cells were represented by red fluorescence, while all cardiac myocytes were represented by blue fluorescence. TUNEL Positive Ratio of myocardial cell apoptosis * Compared with the WT Con $P < 0.01$ Δ Compared with the WT DOX $P < 0.05$ # Compared with the WT DOX $P < 0.05$ Data are expressed as mean $\pm$ SEM and analyzed by using unpaired student t-test.

Figure 6

Changes of p-AMPK / AMPK α 2 protein levels in mouse myocardium

(a) Color ultrasound image of mouse heart

(b) EF(%)

(c) FS(%)

Figure 7

EF and FS test results in each group

Notice：*Compared with the Con， $P<0.05$ ；** Compared with the DOX, $P<0.05$ ；Data are expressed as mean $\pm$ SEM and analyzed by using unpaired student t-test.

Figure 8

Detection results of serum myocardial injury indexes in mice

Notice：*Compared with the Con， $P<0.05$ ；** Compared with the DOX, $P<0.05$ ；Data are expressed as mean $\pm$ SEM and analyzed by using unpaired student t-test.

(b) PDH test results

(c) P-PDH test results

Figure 9

Detection results of myocardial PDH and P-PDH in mice in AKO group

Notice: *Compared with the WT $P < 0.05$

(a) Expression of PDH and P-PDH in each group

(b) PDH test results

(c) P-PDH test results

Figure 10

AMPK overexpression mouse myocardial PDH and P-PDH test results

Notice: *Compared with the Con, $P < 0.05$

(a) The detection result of LC3B-II in the total components of myocardial cells

(b) LC3B-II Mitochondrial component detection results

(c) LC3B-II in the cytoplasmic component test results

Figure 11

The expression results of LC3B-II protein of each component of myocardial cells in the two groups of mice

Notice: **Compared with the WT +pepA/E64d $P < 0.01$

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