Peer Review File

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Reviewer A

Comment 1: The manuscript entitled "Uric acid preconditioning suppresses doxorubicin induced JNK activation and Cx43 phosphorylation and cardiotoxicity via an AMPK-SHP2 signaling pathway activation" has been revealed that uric acid preconditioning significantly upregulated SHP2 expression and inhibited doxorubicin-induced phosphorylation of gap junction protein, Cx43, to alleviate doxorubicin-induced cardiotoxicity. The experimental techniques are well executed and evidenced in both cell culture and animal models. The only issue is the manuscript need English language correction after which the manuscript is suitable for publication.

Recommendation: Accept

Reply 1: Thank you for your suggestions. We have employed a professional English editing service (AME Editing Service, http://editing.amegroups.cn) to re-check and modulate the English for the previous version. (The certificate of English editing is below).

Changes in the test: The changes with blue words is in the revision version.

Reviewer B

Comment 1: Authors present data on the protective effect of uric acid against doxorubicin toxicity using in vivo (mice) and in vitro (H9C2 myocardial cells) models. It is suggested that the protective effect of uric acid is due to the activation of AMPK, with the subsequent modulation of the AMPK-SHP2-JNK-Cx43 signaling pathway. I suggest an extensive revision of English for the precise presentation of the experimental models, results and discussion. Inaccuracies detected in the text make it difficult to understand.

Reply 1: Thank you for your suggestion. We have carefully revised the manuscript and a professional English editing service have helped us re-check the grammar and spelling.

Changes in the test: The changes with blue words was showed in the revision version.

Comment 2: There is the information in the background section of the abstract that "Doxorubicin-associated cardiotoxicity is reportedly closely related to aberrant adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) signaling after chemotherapy." This is not mentioned in the introduction section. At least one reference should be cited for the above statement in the

introduction of the manuscript.

Reply 2: Thank you for your suggestion. We have carefully revised the manuscript and added some related references in the manuscript.

Changes in the test: Page 6, line111-113

Comment 3: Interestingly, data displayed in figure 4 (results section) show that doxorubicin did not have an effect on pAMPK in both experimental models (in vitro and in vivo). A sentence in the discussion section of the manuscript (lines 279-280) directs the reader to a wrong information about the effect of doxorubicin on ATP level, ATP/ADP ratio and AMPK activity reported in reference 22. The sentence is "Firstly, doxorubicin can significantly reduce the ATP level accompanied by decreased ATP/ADP ratio, which in turn decreases the activity of AMPK (22)." Reference 22 does not show the effect of doxorubicin on ATP level, ATP/ADP ratio or AMPK activity. It shows that ATP level and ATP/ADP ratios are altered in cell culture depending on the cell confluence and that the cell confluence can affect the cell sensitivity to chemotherapeutic drugs, such as doxorubicin. Nothing is said about AMPK in this reference. The next sentence (lines 281-282) in the discussion section is "These observations ultimately led us to reasonably believe that doxorubicin-induced cardiotoxicity might be AMPK-related". Which observations? Observations from reference 22 cannot lead to this belief. This sentence calls into question the sentence placed at the beginning of the abstract which says that "Doxorubicin-associated cardiotoxicity is reportedly closely related to aberrant adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) signaling after chemotherapy."

Reply 3: Thank you for your advice. We are sorry for the improper citation of references; we have reviewed the relevant literature again and cited the reference in discussion section. Additionally, we also re-statistical analysis the result (figure 4), and found that the levels of p-AMPK were decreased in doxorubicin-treatment group. Although there is some discrepancy in the literature with respect to AMPK activation expose to doxorubicin treatment (1-3). One factor accounting for opposing findings across different studies may be the vast array of model systems (e.g. mice, rats, cardiomyocytes, mouse embryonic fibroblast) and treatment schemes (ip, iv, single dose, chronic weekly dose, low dose, high dose), most studies agree with cardiac AMPK inhibition in response to doxorubicin.

Changes in the text: Page 6, line111-113, Page 16-17 line 339-342

Comment 4: There is a mixture of the experimental models (in vitro and in vivo) in the methods section of the abstract. It is important to make it clear that two models were used (mice and myocardial cell culture) and that the results and conclusions are based on these two models. The results and conclusions are

presented in the abstract without a clear separation of what was found in the mice model and in the cell culture model.

Reply 4: Thank you for your comments. We have carefully revised the abstract section, and described the methods and the results as clearly as possible.

Changes in the text: Page 3-4, line 42-59.

Comment 5: Animal treatment is not clear in the Materials and Methods section of the manuscript. It is said that the acute cardiotoxicity was induced by a single i.p. dose of doxorubicin (lines 119-120). Different doses of uric acid and allopurinol were intragastrically administered one day before doxorubicin treatment (lines 121-122). But the last sentence (lines 123-125) is "All these agents were administered in a volume of 10 ml/kg for 8 consecutive days, the mortality rate and weight changes of mice were recorded every day."

Reply 5: Thank you for your comments. We have carefully revised the Materials and Methods section of the manuscript.

Changes in the text: Page 7-8, line 140-148.

Comment 6: Uric acid was administered to the animals in the doses of 62.5, 125 and 250 mg/kg. Cells were exposed to uric acid in the concentrations of 1.25, 2.5 and 5 mg/dL. For comparison of the effects between models, it is important to quantify the plasma uric acid concentrations in the animal model. It is also important to present the micromolar concentrations of uric acid in the cell culture medium, besides the mg/dL, for comparison to the concentrations of the other added compounds.

Reply 6: Thank you for your comments. In order to prevent uric acid being metabolized by uricase in the body, the mice were injected with Oteracil potassium (an inhibitor of uricase) 5 min before uric acid treatment. On the 5^{th} day after modelling, we also detected the level of uric acid in the plasma of the mice, the results showed that the uric acid level in uric acid-treated mice is higher than control group of mice. The result was showed in Figure 2H. In this study, the culture medium does not contain uric acid

components, and the cells are rarely produced uric acid in the process of cultivation. Therefore, we think it is not necessary to detect the level of uric acid in cell culture medium.

Changes in the text: Page 8, line 144-146.

Comment 7: Data of the phosphorylated molecules (pAMPK, pJNK, pSHP2) are presented without the corresponding non-phosphorylated pools. In general, the ratio phosphorylated/non-phosphorylated is presented to highlight effects on the activity.

Reply 7: Thank you for your comments. We have showed the ratio phosphorylated p-AMPK/AMPK, p-SHP2/SHP2 and p-JNK/JNK in Figure 3 and Figure 4 in the revision versions.

Changes in the text: The ratio phosphorylated p-AMPK/AMPK, p-SHP2/SHP2 and p-JNK/JNK in Figure 3 and Figure 4 was shown in the revision versions, and some modifications showed in Page 12 line 238-250 and Page 25-26 line 524-541.

Comment 8: I did not find the effect of the SHP2 inhibitor on the heart in vivo, as suggested by the sentence (lines 231-233): “As Fig. 4C shown, shp099 (SHP2 Inhibitor) can abolish the reduction of doxorubicin-induced phosphorylated JNK by uric acid ($P < 0.001$;), and the protective effect of uric acid on the heart in vivo.” Figure 4C shows data obtained in cell culture experiments. The following sentence (lines 246-248) gives the wrong idea that the cell culture model used is a heart in vitro. “In contrast, the inhibition of SHP2 repealed the reduction of doxorubicin-induced phosphorylated JNK and Cx43 by uric acid and repealed the protective effect of uric acid on the heart in vitro.”.

Reply 8: Thank you for your comments. We are sorry for the mistake, and we accidentally wrote "in vitro" as "in vivo" in the previous versions. We have revised the section of result and discussion.

Changes in the text: Page 13 line 273, Page 14 line 280.

Comment 9: Many controls are missing in figure 4C and 4D.

Reply 9: Thank you for your comments. We have revised the Materials and Methods section, results

section, figure and figure legends section, and you can see it in the revised versions.

Changes in the text: We have revised the Materials and Methods section, results section, figure 4C and 4D, and figure legends section, and you can see it in the revised versions.

Reviewer C

General Comments:

Comment 1. Title: The title is suggested to be reformatted as:

"Uric acid preconditioning alleviated doxorubicin induced JNK activation and Cx43 phosphorylation associated cardiotoxicity via activation of AMPK-SHP2 signaling pathway"

Reply 1: Thank you for your comments. We accepted the reviewer's suggestions.

Changes in the text: Page 1 line 1-3.

Comment 2. Abstract: Poor written English! Please seek the help of some English speaking experts to reformat this section.

Reply 2: Thank you for your reminder. We have utilized a professional English editing service (AME Editing Service, http://editing.amegroups.com/) to re-check and polish the manuscript for the previous version.

Changes in the text: We have revised the manuscript and showed it with blue word in the revised versions.

Comment 3. Abstract/Line 41: Show the dose and concentration of doxorubicin and uric acid, the administration method, how many times per day?!

Reply 3: Thank you for your reminder. We have revised the abstract section and you can see it in the revised versions.

Changes in the text: Page 3-4 line 42-59

Comment 4. Abstract/Line 44: The written English has some space to improve! For example, Line 44: "Doxorubicin effects on....."

Reply 4: Thank you for your reminder. We have made modifications as you suggested.

Changes in the text: Page 3 line 42-50

Comment 5. Title: The title is suggested to be reformatted as:
"Uric acid preconditioning alleviated doxorubicin induced JNK activation and Cx43 phosphorylation associated cardiotoxicity via activation of AMPK-SHP2 signaling pathway"

Reply 5: Thank you for your reminder. We accepted the reviewer's suggestions.

Changes in the text: Page 1 line 1-3.

Comment 6. Section "Results": Use past tense to describe your results!

Reply 6: Thank you for your reminder. We have made modifications as you suggested.

Changes in the text: We have revised the manuscript with blue word in the revised versions.

Comment 7. The number of animals use for experiment was only 10 for each group. I have much concern about the fact that "a true accuracy can not be approached". My experiences have let me recognized the mortality caused by treatment with Doxorubicin was very random.

Reply 7: Thank you for your reminder. We also repeated the experiment, and found that the death rate of doxorubicin-treatment group of mice was high on the eighth day. One factor accounting for different findings across different studies may be the vast array of model systems (e.g. mice, rats) and treatment schemes (ip, iv, single dose, chronic weekly dose, low dose, high dose), which are employed to assess mortality caused by treatment with doxorubicin. Your suggestions will be considered completely in our further study.

Specific Comments:

Comment 8. Line 41: Show the dose and concentration of doxorubicin and uric acid, the administration method, how many times per day?!

Reply 8: Thank you for your reminder. We have revised the abstract section and you can see it in the revised versions.

Changes in the text: Page 3-4 line 42-59.

Comment 9. Line 118-119: Uric aci and alloprinol are only slightly soluble or almost insoluble in water, the use of CMC-Na is to suspend these drugs!

Reply 9: Thank you for your reminder. We have revised the section of Materials and Methods in line 138-140. The uric acid and allopurinol were suspended in 0.5 % CMC-Na solution, and before given it to mice, it would be resuspended by slightly concussion.

Changes in the text: Page 7 line 138-140.

Comment 10. It is unbelievable that doxorubicin at 20 mg/kg could induce acute cardiotoxicity. My experiences told me that before having induced the symptom of cardiotoxicty, the kidney would have been damaged! In particular, by i.p.

Reply 10: Thank you for your reminder. Actually, a single intraperitoneal injection of doxorubicin (15mg/kg or 20mg/kg) has been used by many researchers to establish the acute cardiotoxicity model of doxorubicin (4-7). Additionally, previous studies have also found that doxorubicin has a very high affinity by cardiolipin, a phospholipid specie mainly present in mitochondrial membranes of heart, which results in the accumulation of doxorubicin inside cardiac cells (8,9). In this study, we were referred to relevant literature to establish an acute cardiotoxicity model of doxorubicin, and the results showed that mice began to die on the 5^{th} day after doxorubicin administration, and both electrocardiogram and myocardial enzyme testing showed that a single intraperitoneal injection of 20 mg/kg doxorubicin could induce cardiac disease.

Comment 11. Line 253-255: I could not catch the idea of the authors!

Reply 11: Thank you for your reminder. As we can see, uric acid is a product of the catabolism of purine nucleotides, previous studies have found that excessively high levels of uric acid increase the risk of heart disease and gout (10-12), however, at the same time, uric acid also has a strong antioxidant effect, and many studies have also shown that uric acid can achieve cell protection through its antioxidant activity (13-16). In addition, a recent study found that febuxostat，a drug to lower uric acid, which can increase the

incidence of cardiovascular disease in patients (17-19). Here, we just want to express the view that too high or low uric acid is not a good thing, and blindly reducing uric acid levels may not benefit from it.

Changes in the text: Page 15 line 299-301.

Comment 12. Please sketch a graphic summary of your experimental results and presnt in section "Discussion"

Reply 12: Thank you for your comments. Based on our results, we draw a summary diagram and explain how uric acid plays a protective role in the heart through the AMPK-SHP2-JNK-Cx43 signalling axis in figure 5.

Changes in the text: Page 14 line 292. Page 26-27 line 555-562.

Comment 13. Figure1 to Figure 4: Poor written English! Please reformat the figure legends for these figures.

Reply 13: Thank you for your reminder. We have revised the figure and figure legends.

Changes in the text: Page 24-26 line 504-554.

Comment 14. Figure 4B: The level of p-AMPK was higher in group (UA5+DOX1) than group (UA5+DOX0), why? This must be discussed!

Reply 14: Thank you for your reminder. In fact, there is some controversy about the effect of doxorubicin on AMPK, but most studies support the idea that doxorubicin can reduce the activity of AMPK in rat heart tissue (2,20). In this study, our result also supports the idea, therefore, we replace the western blot image, and re-statistical analysis found that the level of p-AMPK between (UA5+DOX1) group and (UA5+DOX0) group showed no statistically significant difference in figure 4B.

Changes in the text: We have replaced and re-statistical analysis the figure 4B, and there was no statistically significant difference.

Comment 15. Line 208-209: Your result in fact was not “p-AMPK dependent”. As p-AMPK was not added externally! This sub-caption must be reformatted!

Reply 15: Thank you for your reminder. In this study, our results showed that uric acid preconditioning could significantly increase the level of p-AMPK in vivo and in vitro, and the AMPK agonist (AICAR) could mimic the protective effect of uric acid in vitro, whereas pretreatment with AMPK inhibitor (compound C) could reverse these effects. In this study, we have re-statistical analysis of the results and showed it in figure 3-4.

Changes in the text: we have re-statistical analysis of the results and showed it in figure 3-4.

Comment 16. All through your text: Use italized lower case “p” to indicate the statistical CL. Don’t use upper case “P”!

Reply 16: Thank you for your reminder. We have made modifications as you suggested.

Changes in the text: The upper case “P” have been replaced to lower case “p” in the revised versions.

Reviewer D

Comment 1. Introduction: Extremly concise describing only the literature results about the doxorubicin-induced cardiotoxicity. The nature of the problem should emphasize other parts of purine metabolism, uric acid production and experimental results about uric acid two-face properties. It should set other works in the context of similar research, but opposite results, citing relevant references.

About uric acid, the sentence:
Since doxorubicin-induced cardiotoxicity is associated with both ROS-induced myocardial
injury and inflammation-induced conduction disorder (8), it is necessary to discover a safe and stable compound that can ameliorate both the adverse effects of doxorubicin.

Seems not to be unique literature report:

Part I
The findings of:
1. Yan M, Chen K, He L, Li S, Huang D, Li J. Uric Acid Induces Cardiomyocyte Apoptosis via Activation of Calpain-1 and Endoplasmic Reticulum Stress. Cell Physiol Biochem. 2018;45(5):2122-2135.

doi:10.1159/000488048...suggesting that UA induces cardiomyocyte apoptosis through activation of calpain-1 and ER stress, the mechanisms of hyperuricemia-associated cardiovascular risks....Or

2. Kocic G, Sokolovic D, Jevtovic T, et al. Short communication: Effect of commercial or depurinized milk diet on plasma advanced oxidation protein products, cardiovascular markers, and bone marrow CD34+ stem cell potential in rat experimental hyperuricemia. J Dairy Sci. 2014;97(11):6823-6827. doi:10.3168/jds.2014-8556

...where experimental hzperuricemia was cardiotoxic and allopurinol amelioreted it.

3. Sautin YY, Johnson RJ. Uric acid: the oxidant-antioxidant paradox. Nucleosides Nucleotides Nucleic Acids. 2008;27(6):608-619. doi:10.1080/15257770802138558

...where the pro-oxidative effects of uric acid that occur in cardiovascular disease and may have a contributory role in the pathogenesis of these conditions

4.Sagor MA, Tabassum N, Potol MA, Alam MA. Xanthine Oxidase Inhibitor, Allopurinol, Prevented Oxidative Stress, Fibrosis, and Myocardial Damage in Isoproterenol Induced Aged Rats. Oxid Med Cell Longev. 2015;2015:478039. doi:10.1155/2015/478039

These articles or other with similar findings should be cited.

Reply 1: Thank you for your comment. In this study, the aim is to investigate whether uric acid preconditioning could alleviate doxorubicin-induced cardiotoxicity and whether its mechanism involved AMPK signaling.

In the introduction section, we introduced the harm of the doxorubicin-induced cardiotoxicity and its possible mechanism, and based on the previous literature research and the relevant experimental results, we found that the change of gap junction (Cx43), which would lead to cardiac conduction abnormalities and the caused cardiomyopathy. Then, we take this as an entry point, and further propose a hypothesis that regulates energy-related molecules, AMPK, as the targets to promote the production of negative regulatory substances in the body to resist doxorubicin-induced cardiotoxicity.

In this study, uric acid is the key drug molecule. So, in the second paragraph of the discussion section, we are focused on the two sides of uric acid and discuss its cytoprotective effect as a powerful antioxidant. And we also cited relevant reference which is recommended by you.

Changes in the text: We have revised the manuscript with blue words in Page 13-15 line 293-310 in the revised versions.

Part II

The nature of the problem should be emphasized in light of Cell energy crisis followed by the fall in ATP level, which dramatically increases total purine degradation, activation of XO and urate production, according to the Atkinson equation of Adenylate energy charge:

AEC = (ATP + 1/2ADP): (ATP + ADP + AMP)

The article concerns the adenylate salvage pathway, what about the activation of AMP degradation pathway? It is very important in organ damage:

Boban M, Kocic G, Radenkovic S, et al. Circulating purine compounds, uric acid, and xanthine oxidase/dehydrogenase relationship in essential hypertension and end stage renal disease. Ren Fail. 2014;36(4):613-618. doi:10.3109/0886022X.2014.882240

Comment 2. Methods: provides sufficient details of the experiment and statistical test

Reply 2: Thank you for your comment. We have revised the section of Materials and Methods and you can see it in the revised versions.

Changes in the text: We have revised the section of Materials and Methods with blue words, in Page 6-10 line118-197.

Comment 3. Results: give the main findings and outcomes of your study.

Reply 3: Thank you for your comment. We have revised the section of Results and you can see it in the revised versions.

Changes in the text: We have revised the section of results with blue words in Page11-14 line 208-282, and you can see it in the revised versions.

Comment 4. Discussion: Should discuss the significance of the results and compare with the opposite results. Athe authors should give a clear reccommendation why they stand on the standpoint that uric acid should be useful in therapy. What about potential late consequences and urate calculosis, or urate depositions in the kidney or blood vessels and atherosclerosis. If no, the article may point: one toxic effect

should be exchanged with other...

Reply 4: Thank you for your comment. Uric acid is a product of the catabolism of purine nucleotides, although previous studies have found that excessively high levels of uric acid increase the risk of heart disease and gout (10-12), it also indicated that uric acid has a powerful antioxidant effect (13,14). Although persistent hyperuricemia may induce the apoptosis of cardiomyocyte through activation of calpain-1 and endoplasmic reticulum stress (21), and repeated administration of uric acid may be unfavorable to joint and renal disease, it is also difficult to determine an absolute serum concentration beyond which risk is significantly increased. Additionally, patients with arthritis and tumor lysis syndrome, even the concentrations of uric acid beyond 1,200 μM does not necessarily cause gout (22). Moreover, renal impairment in the presence of chronic hyperuricemia is more often attributable to other factors, such as hypertension or diabetes, rather than serum uric acid concentration itself. Thus, it seemed that rising uric acid levels is not a completely bad thing. In this study, we found that a uric acid-lowering medicine, allopurinol, could increase the mortality rate of doxorubicin-treatment mice. In addition, we also found that uric acid preconditioning significantly increased the survival rate and body weight of mice, and reduced the level of AST, CK-MB and LDH relative to only doxorubicin-treated mice. Additionally, studies have found that uric acid does not cause tumor drug resistance to doxorubicin (23). Based on the above results, we speculated that the rise in endogenous uric acid levels may have a certain protective effect on the heart.

Changes in the text: we have revised the discussion section in Page 15 line 301-310.

Comment 5. Conclusion: This section should highlight the new explanations to oppose the novelity and significance of the work, which would be able to repeal a hundrid opposite articles.

Reply 5: Thank you for your comment. We have revised the section of conclusion and you can see it in the revised versions.

Reviewer E

Comment 1: Abstract & Methods:

-It seems that uric acid was administrated only one day before doxorubicin in abstract. However, in methods it was described that uric acid was administrated for 8 consecutive days. Please describe clearly how long uric acid was administrated.

Reply 1: Thank you for your comment. We have revised the Abstract & Methods section. Different concentrations of uric acid (62.5, 125, and 250 mg/kg) were firstly intragastrically (*i.g.*) administered into mice one day before doxorubicin treatment, and then continuously given the drugs for 8 days, every 24 hours.

Changes in the text: We have revised the Abstract section in Page 3 line 42-46, and Methods section in Page 8 line 142-144.

Comment 2. In this study uric acid were administrated into mice intragastrically. Uric acid is metabolized to allantoin immediately by uricase in liver in mice. And serum uric acid level in mice is very low around at 0.5 mg/dl. In general, to investigate the effect of uric acid in vivo in mice, hyperuricemia model mice, which uricase is inhibited by administration of oxonic acid or knocked out genetically, are used for experiments.

Does the protective effect on DOX cardiotoxicity really result from uric acid itself? There is a possibility that the effect was caused by allantoin. The authors need to show that serum or plasma uric acid level is higher in the mice administrated uric acid. If possible, the uric acid level in cardiac tissue should be shown, too.

Reply 2: Thanks for your comment. In fact, in order to prevent uric acid being metabolized by uricase in the body, the mice were injected with Oteracil potassium (an inhibitor of uricase) 5 min before uric acid treatment. On the 5^{th} day after modelling, we also detected the level of uric acid in the plasma of the mice, the results showed that the uric acid level in uric acid-treated mice is higher than control group of mice (Fig. 2I). Therefore, we reasonably speculated that uric acid could protect against doxorubicin-induced cardiotoxicity in mice.

Changes in the text: We have revised the Methods section in Page 8 line 144-146, and added the results in figure 2I.

Comment 3: Thank you for your comment. As for Figure 1A, please insert a symbol to represent the significant difference. The method for statistical analysis should be described in Figure legend.

Reply 3: Thank you for your comment. We have revised the figure 1A, and the method for statistical analysis have been described in Figure legend in line 505-508.

Changes in the text: We have revised the figure 1A, and the method for statistical analysis have been described in Figure legend in Page 24 line 505-508.

Comment 4: Figure 2:

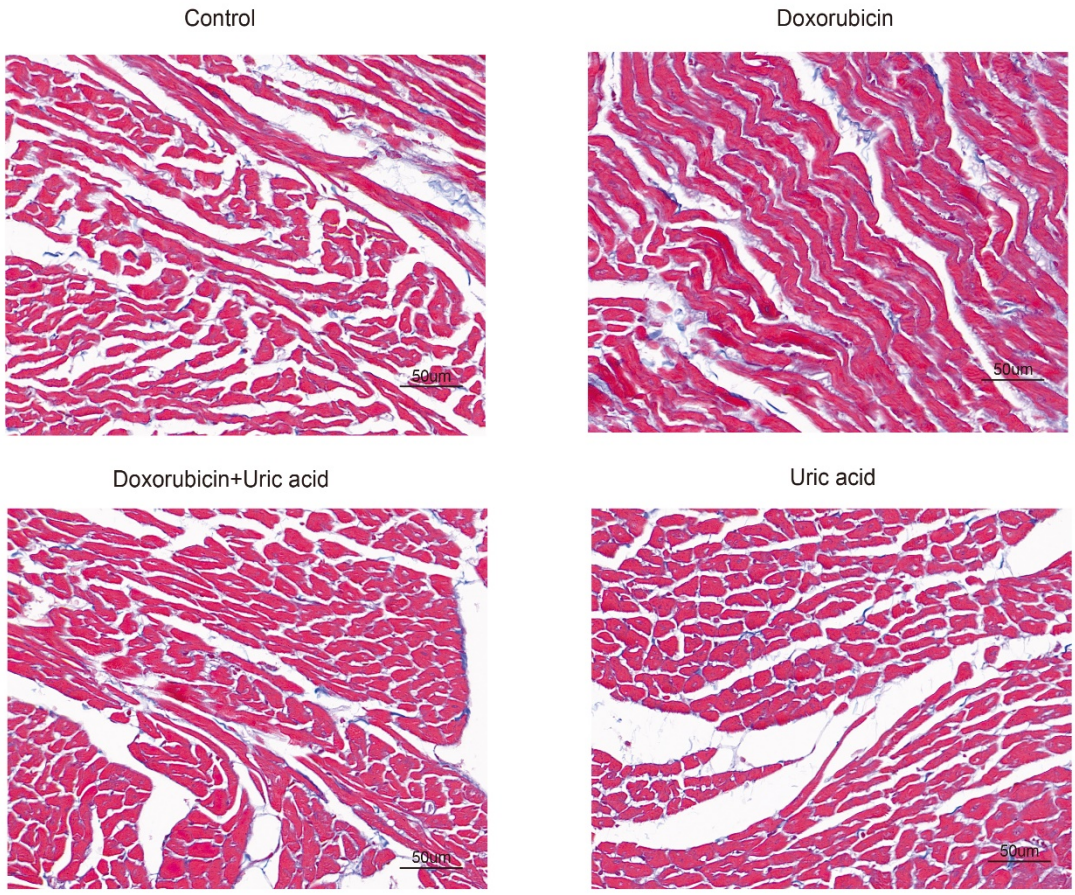
They were very clear data to understand the effect of uric acid on DOX cardiotoxicity. From ECG data uric acid alleviate the cardiac conduction by DOX. And, elevation of cardiac enzyme levels was also suppressed in mice administrated uric acid. It may indicate that uric acid was effective against both conduction disorders and myocardial injury.

-Do you have any pathology which show that DOX cardiotoxicity was attenuated by administration of uric acid?

-The labels of Y axis in panel G and H may be the opposite.

Reply 4: Thank you for your comment. We had performed the experiment to investigate whether doxorubicin administration results in structural changes and myocardial damage in the mouse heart. On the 5th day after modeling, the heart tissues were used to Masson staining. The result shown that there were no different in each group, and no obvious myocardial fibrosis was found in the doxorubicin treated group; Moreover, uric acid treatment group also had no significant effect on the myocardial cell structure of mice (see image below). Therefore, we believe that the cause of death in doxorubicin-treated mice may cause by severe abnormal arrhythmias. In this study, we focused on uric acid to reverse arrhythmia caused by doxorubicin.

Moreover, we have revised the labels of Y axis in panel G and H in figure 2.



Representative images of Masson staining in myocardial sections. Scale bar: 50 μm.

Changes in the text: We have revised the labels of Y axis in panel G and H in figure 2.

Comment 5: Figure 3:

-Please show not only p-SHP2 and p-AMPK but also total SHP2 and total AMPK in western blot. I am interested in the effect of uric acid to p-AMPK which is the activation of phosphorylation of AMPK and/or the elevation of expression of total AMPK in vivo.

Reply 5: Thank you for your comment. we have performed the experiment to investigate the effect of uric acid on total AMPK expression level in vitro and in vivo. The results indicated that uric acid did not affect the level of total AMPK expression in vitro and in vivo.

Changes in the text: We have replaced the figure 3 in the revised versions.

Comment 6: Also, please show total Cx43 and JNK in Figure 3D and 4A. The transcription of total Cx43 also influences on the conduction disorder. Is there any change of total Cx43 by administration of uric acid?

Reply 6: Thank you for your comment. Previous study has shown that chemotherapy may cause down-regulation of Cx43 total protein and upregulation of its phosphorylated form, p-Cx43 (24,25). Additionally, the reduction of gap junctions (Cx43) would lead to reduce intercellular coupling, thereby lowering conduction velocity (26). In this study, we investigated the effect of uric acid on doxorubicin-induced Cx43 gap junction, the results shown that uric acid significantly reduced doxorubicin-induced the phosphorylation of Cx43, but upregulated the total Cx43 protein in mice.

Changes in the text: We have replaced the figure 3 D and 4A in the revised versions.

Comment 7. As for Figure 3C and 3D, the fold change of p-SHP2 and p-JNK in picture looked much higher than that in graph. The authors should present the picture which is less discrepancy with the data in graph.

Reply 7: Thank you for your comment. We have replaced the images and re-statistical analysis the results.

Changes in the text: We have replaced the images and re-statistical analysis the results, and showed it in figure 3C and 3D in in the revised versions.

Comment 8. The effect of uric acid on AMPK is controversial. Some studies have reported that uric acid activates AMPK as well as the manuscript, but other studies reported the opposite results. Please mention these previous reports about the association of AMPK with uric acid in discussion.

Reply 8: Thank you for your comment. We have revised the discussion section.

Changes in the text: We have revised the discussion section and showed it in Page17 line 354-355.

Comment 9: As for Figure 4D, the authors showed the protective effect of uric acid-AMPK pathway on the cytotoxicity of H9C2 cells, but it was a little confusing data. Please clarify the end point of this experiment.

Maybe the cell index is influenced by not only cell number but cell size, cell morphology or strength of cell attachment. The cell index of the groups of "Dox + UA" and "AICAR" is likely to be higher than that of control group during the early phase. I wonder that the result was influenced not by the cytotoxicity but by the change of cell morphology or size. The other cytotoxicity assay of H9C2 cells by doxorubicin, such as the rate of cell viability or apoptosis, should be shown, too. If possible, it should be shown the effect of uric acid-AMPK pathway on DOX-cardiotoxicity in vivo. For example, please show that the suppression of AMPK activity by administration of compound C in DOX + UA mice results in the attenuation of effect of uric acid on DOX-cardiotoxicity (as for mortality rate or QTc prolongation).

Reply 9: Thanks for your comment. The iCELLigence™ real-time cell analyzer (RTCA) can be used for the label-free real-time monitoring of the cell proliferation, viability, invasion and cytotoxicity. In a typical run, upon cells' incubation on the arrayed gold microchips, the produced electrical impendence reflecting the physiological status of cells such as cell proliferation and viability was continuously monitored (26-28). Without the use of labeled dyes, RTCA permits a direct and continuous measurement of cells under physiological conditions (29), and overcomes the limitation of end-point data and incompatibility with orthogonal assays (30,31). The system obtains real-time results that reflect the adhesion, proliferation, apoptosis, propagation and morphologic changes of cells over long periods of time.

In this study, 1×10^4 well of H9C2 cells were seeded into 16-well microtiter plates (with a gold microelectrode biosensor array) and cultured for 24 hours with complete culture medium containing FBS, and then treated with AMPK agonist or inhibitor, uric acid, respectively, followed by doxorubicin treatment for 24 hours. During this time, RTCA system effectively helped us to continuously detect the cell growth status in real time. As can be seen from the figure 4D, compared with the doxorubicin group, AICAR and uric acid treatment obviously promote cell proliferation and viability.

Previous study has found that doxorubicin could induce apoptosis in H9C2 cardiomyocytes (32), and it has also been found that uric acid can inhibit apoptosis (33). In this study, our result showed that uric acid activated AMPK in a time- and concentration-dependent manner, and AMPK agonist (AICAR) could mimic the protective effect of uric acid on cardiomyocytes in vitro, while preconditioning with compound C could reverse these effects. Moreover, previous studies have found that uric acid elevation may be a protective response, capable of opposing the harmful effects of free-radical activity and oxidative stress (16), and it has also been found that uric acid can activate AMPK (34). Base on these results, we

reasonably speculated that uric acid protected against doxorubicin-induced cardiotoxicity in an AMPK-dependent manner. Certainly, further studies will be conducted on AMPK gene knockout mice.

Changes in the text: please see the revised manuscript for details.

Comment 10: Please mention more clearly the relationship of the AMPK-SHP2-JNK-Cx43 pathway with DOX-cardiotoxicity in discussion. For example, how was the ECG in Cx43-KO mice in previous studies? Did it have QTc prolongation or ST change as well as the ECG of DOX-cardiotoxicity? If there was any reports that an AMPK activator was used for DOX-cardiotoxicity, please mention that.

Reply 10: Thanks for your comment. Previous evidences have indicated that knock down of the carboxyl terminal (CT) domain of Cx43 would lead to an increase e in infarct size and increase susceptibility to arrhythmias following acute coronary occlusion (35). In addition, AMPK activator, such as resveratrol, metformin and AICAR, have also been reported a protective effect in doxorubicin-induced cardiotoxicity (3,36-38).

Changes in the text: We have revised the discussion section in Page 16-17 line 331-334, and line 339-341.

For editor

The manuscript is an interesting and seems to have sufficient novelty.

However, it has several problems as described in comments for authors. Especially, I have a question whether the mouse model was valid. The mice were administrated uric acid intragastrically, but in general uric acid is metabolized to allantoin immediately in mice. And whether uric acid suppressed DOX cardiotoxicity via AMPK-SHP2-JNK-Cx43 pathway also needs further consideration.

I think that these two major points at least need to be clear for acceptance.

Reply: Thanks for the editor's question. Firstly, a single intraperitoneal injection of doxorubicin (15mg/kg or 20mg/kg) has been used by many researchers to establish the acute cardiotoxicity model of doxorubicin (4-7). In this study, we were referred to relevant literature to establish an acute cardiotoxicity model of doxorubicin. And our results showed that mice began to die on the 5th day after doxorubicin administration, and both electrocardiogram and myocardial enzyme testing showed that a single intraperitoneal injection

of 20 mg/kg doxorubicin could induce cardiac disease.

Secondly, in order to prevent uric acid being metabolized by uricase in the body, the mice were injected with Oteracil potassium (an inhibitor of uricase) 5 minutes before uric acid treatment. On the 5th day after modelling, we also detected the level of uric acid in the plasma of the mice, the results showed that the uric acid level in uric acid-treated mice is higher than control group of mice (Fig. 2I). Therefore, we reasonably speculated that uric acid could protect against doxorubicin-induced cardiotoxicity in mice.

Thirdly, our results showed that uric acid preconditioning not only inhibited doxorubicin-induced cardiotoxicity, but also could induce the activation of AMPK and SHP2, and reduce the level of p-JNK and p-Cx43 in vitro and in vitro; pretreatment with AMPK agonist (AICAR) could mimic the protective effect of uric acid on cardiomyocytes in vitro, and increased the level of p-SHP2 in vitro, while preconditioning with AMPK inhibitor (Compound C) could reverse these effects, moreover, pretreatment with shp099 (a SHP2 inhibitor) could abolish the reduction of doxorubicin-induced phosphorylated JNK by uric acid.

Connexins 43 (Cx43), integral membrane proteins that form gap junctions enable the direct cytoplasmic exchange of information and substances between adjacent cells and contribute to cardiac conduction. Studies have shown that doxorubicin could induce the activation of JNK (39,40), and then caused the down-regulation of Cx43 total protein and upregulation of its phosphorylated form, p-Cx43 (41), and resulting in a significant reduction of conduction velocity (42), and reduced Cx43-mediated cell-to-cell communication. Moreover, our previous research has also found that activated AMPK could inhibit inflammatory response through suppressed the activation of JNK (43). Therefore, we speculated that uric acid preconditioning alleviated doxorubicin-induced cardiotoxicity through the AMPK-SHP2-JNK-Cx43 signaling pathway. Certainly, we need to further confirm the mechanism through gene knockout mice.

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