Author's response to reviews

Title: DanQi Pill protects against heart failure through the arachidonic acid metabolism pathway by attenuating different cyclooxygenases and leukotrienes

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Author's response to reviews: see over
First, we really appreciate your and the reviewers’ helpful comments and valuable suggestions. According to the comments of the reviewers, we have made corrections and modifications as follows:

**Reviewer 1:**

**MINOR COMMENTS:**

1. What are the compounds identified in DQP? Is it published elsewhere? Did the authors use all the compounds in the drug prediction target?

This is a very important question. In present, compounds in *salvia miltiorrhiza* and *panax notoginseng*, which contained in DQP, are studied deeply. For example, recently the transcriptome and genome of *S. miltiorrhiza* have been significantly recovered [1]. The research prospect of *S. miltiorrhiza* as medicinal model plant in TCM was also discussed, including biosynthesis of active components and their genetic regulation [2]. The results showed that more than 50 compounds are contained, which are rich in phenolic acids and different levels of tanshinones [3]. The same researches have been carried on the *panax notoginseng*, the main compounds are *panax notoginseng* saponins and flavonoids with more than 40 subtypes [4-5]. In our paper, all the compounds contained both in *salvia miltiorrhiza* and *panax notoginseng* which have been identified so far, are included to predict their potential targets.


2. The logic behind concentrating on arachidonic acid metabolism pathway is not clear since it is not the top pathway identified in the analysis.

Indeed, arachidonic acid metabolism pathway (AA pathway) is not the top pathway identified in the analysis. The reasons we selected this pathway are as follows: among the 7 pathways, the 1, 4 and 5 pathways were the common metabolites pathway that existed in almost all disease, while the 2, 3 and the AA pathway are considered as the critical mechanism of heart failure (HF) [1-2]. The agonists of 2 and 3 pathways have already been proved to have definite efficacy on HF [2], and some previous studies have demonstrated the salvia miltiorrhiza and panax notoginseng had effects on pathway of 2 and 3[3,4]. Although some drugs especially nonsteroidal anti-inflammatory drugs were developed to target on the AA pathway, they have serve side effects such as increased gastrointestinal and adverse cardiovascular events [5]. In addition, the coverage rate of the AA pathway is highest in our analysis, and the AA metabolism was rarely investigated in HF and DQP’s efficacy, so our original purpose is to validate this pathway thus to provide a complementary and alternative treatment for HF through target on the AA metabolism pathway.


3. A model to explain the pathway affected by treatment would be beneficial.

The model we selected is HF model. In previous study, DQP is commonly prescribed in routine treatment of HF in China. Large-scale randomized and controlled clinical trials have proved that DQP has a definite effect in improving heart function [1]. But its underlying mechanism is undefined. In present study, our predicted targets of DQP indicated that the AA pathway seemed to be the potential mechanism of DQP acting on HF. So the model we need should meet two conditions: the AA metabolism pathway activated with the occurrence of HF. After we checked the reference, we found the LAD-ligated model can fulfill the two conditions which is also repeatable and stable. We also considered the cell model to validate, but there was a technique problem that the cell can’t survive after the addition of the mixture of DQP.


4. Quality of the echo images should improve.

As you suggested, we improved the quality of echo images in our revised manuscript.

5. The pattern of the bands of Fig 3 top panel and Fig 4 top panel look the same. It looks like both are the same. Clarification is needed.

We apologize for this error we made and we appreciate for the suggestions you gave us. I am very sorry for the issue that has arisen during our manuscript preparation. Our mistakes were made during the progress of figures edited. We corrected it and carefully check all the manuscript again to avoid the similar mistakes.

6. Fig 2 - Magnification bar is required.

As you suggested, we added the magnification bar in our revised manuscript.
1. Background

The leading cause of mortality worldwide is ischemic heart disease, not heart failure (http://who.int/mediacentre/factsheets/fs310/en/). I’m sorry, but you have not done your homework with this background section. Roifman, et al., (citation 2), is a systematic review of the association between chronic inflammatory diseases and coronary artery disease; the sentence that cites this study has nothing to do with either chronic inflammatory disease or CAD. Perhaps this citation has been misplaced, although other citations in the background section also appear to be misapplied (eg. citation 1, Panza, et al. is about inducible MI and LV dysfunction, not the leading causes of mortality worldwide). The term effective (paragraph 2) implies both efficacy (preferably demonstrated in large, well done, randomized controlled trials), and the ability to implement the efficacious treatment in real world environments beyond the RCT. Citation 5 appears to be review article on pattern differentiation in Chinese medicine, which seems a bit far-fetched to support the authors claim that ‘some herbal formulas have been proven to be effective’. I suggest that this background section be rewritten with reference to appropriate, primary studies where appropriate.

Thanks for your valuable suggestion. We accept the suggestion, and after carefully reading the related paper, we rewritten this section in our revised paper.

After we checked the website you provided, just as you told us, the leading cause of mortality worldwide is ischemic heart disease, not heart failure. But when we wrote the manuscript, introduction of some references misled us including some newest paper [1-2]. We had corrected it as you suggested. Moreover, we also updated the Roifman, et al., (citation 2) as “Bozkurt B, Mann D L, Deswal A. (2010) Biomarkers of inflammation in heart failure. Heart Fail Rev, 15: 331-341.” to be more accurate, and the sentence also altered. Citation 5: We added a newly published article on Journal of the American College of Cardiology. This article discussed a formula
efficacy on chronic heart failure by a multicenter randomized double-blind parallel-group placebo-controlled study [3]. Interestingly, the formula also contains the same herb-\textit{Salvia miltiorrhiza}, as our current study.


2. Methods

a. There are many acronyms used throughout the manuscript. Please spell them out the first time they are introduced to the reader (eg. PNS, KEGG, SMILES, SPF, PST, etc.). Information on providing names of mouse strains can be found here [http://www.informatics.jax.org/nomen/](http://www.informatics.jax.org/nomen/)

As you suggested, we revised the acronyms in our revised manuscript. Among these acronyms, the “PST” is the type of sector scanner made by Toshiba Company.

b. Please more clearly describe the sham, model, and DQP group. Also state in methods how many rats were in each group and the method used to allocate them to groups.

Thanks a lot for the suggestion. We added the grouping information in the revised manuscript. And the number of rats and their mortality rate were also added as you suggested. All the rats were distributed in different groups randomly.

c. What does the phrase ‘…within a short period of time.’ at the end of 2.2 refer to?

Sorry for the confusion we brought. “Within a short period of time” referred to the time point we took the subsequent detection after the rats were killed. In the revised
paper, we deleted it to avoid confusion.

d. Please avoid non-specific indicators such as ‘… other indicators.’ (Section 2.3, paragraph 1). If the indicators are important, then please list them. As you suggested, we deleted it.

e. What was the PST 65A sector scanner used to do?

The scanner was used to generate two-dimensional images for echocardiography. It can display the heart by constructing a plane image composed of many lines. The signal strength can visualize the cardiac structures, including LVEDs, LVEDd and EF value, etc.

f. Section 2.4 includes ‘dose consideration’ in the section title, however there is no information on the dose used, nor how the choice was made and justified. Indeed, about the “dose consideration”, it appeared in the “2.2 HF model preparation”, namely “The DQP group was treated for 28 days, with the total daily dosage of 1.5 g/kg of the concentrated DQP”. We removed the sentence to this section as you suggested. Based on the recommendation of daily human dosage (20 g/d) and the equivalent conversion between animal and people by body surface area and body weight, dosage of 1.5 g/kg was chosen in the present study.

g. Importantly, how was product integrity independently tested/verified? Which plant parts were used? Was the hot water extraction an infusion or decoction? How was the water?

This is a very important question. First, the parts of roots were used both in *salvia miltiorrhiza* and *panax notoginseng*. Moreover, the DQP we used in this study is Chinese patent medicine (Production lot: 6128006) produced by TongrenTang, a famous pharmaceutical company in China. DQP is widely produced in accordance with the China Pharmacopoeia standard of quality control [1, 2]. Strictly, it is hot water extraction. The water extraction and alcohol precipitation methods were used to
h. Section 2.5.2 begins ‘The tissue….’ This is the first time the reader learns that tissue, other than whole blood, has been collected for analysis (ie. section 2.2 only mentions blood). What are the tissue samples, how and when were they collected? What method was used for protein extraction?

The sample we collected was the heart tissue. The heart was excised and incubated in ice-cold PBS to wash out blood. Each left ventricle was then carefully dissected to remove all the necrotic/scarred zones to keep only the viable myocardium in the marginal zone of the infarct region in model animals. The left ventricular myocardial below ligation bit in sham animals were also dissected. The tissue was homogenized in RIPA buffer (i.e., 50 mM Tris-HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, and 0.1% SDS), and total protein was extracted from this homogenate. We added the information in our revised manuscript as suggested.

i. Section 2.6, Statistical analysis. This section provides very little detail about the very important statistical procedures performed during these studies. Although I am unfamiliar with DrugCIPHER-CS, other genome-wide studies typically need to adjust for false discovery (FDR). How was this done for the current methods? How was the value of 0.1% targets determined? How did you choose the final 7 pathways to be analyzed? What was the prespecified plan for the KEGG analysis? An ANOVA is
appropriate for analysis of the 3 rat groups, but what was the prespecified plan for between-group analyses when the ANOVA was significant (it should be adjusted for multiple testing – if all pairs were tested, a bonferroni corrected p-value would be 0.015 for each individual t-test, for example)? Additionally, a t-test is often used after ANOVA, but there is no mention of how pair-wise groups were tested. There appear to be many ANOVAs – how was multiple testing controlled?

We are sorry that we didn’t describe the bioinformatic approaches in details. As you suggested, we added bioinformatics procedures in the revised manuscript in section of “2.1 Drug–target prediction and analyses”. DrugCIPHER-CS recently presented by Li et al. [1] achieves good prediction performance in our previous study and can infer drug-targets in the genome-wide scale [2]. This method is based on the hypotheses that i) drugs with similar chemical structure usually bind functionally related proteins, and ii) functional relationship between the proteins can be measured by their distance in the protein interaction network. Given a set of known drug (drug space)-target (target space) interactions, for a query drug and a candidate target-gene, drugCIPHER-CS will measure the likelihood of their interaction based on the correlation between the query drug’s structure similarity vector with the drug space and the candidate gene’s functional similarity vector with the target space. For a query compound, drugCIPHER-CS will prioritize the proteins in the protein interaction network (i.e. candidate proteins) according to the order of the decreasing drug-target interaction likelihood, and the candidate proteins with high likelihood will be hypothesized as the potential drug-targets [1].

Here known drug-target interactions are obtained from DrugBank database [3]. We only use those drug-target interactions whose drugs are FDA-approved and have
InChI identifiers [4] and whose targets are human genes/proteins. The chemical structure similarity is calculated based on compounds’ MOLPRINT 2D descriptors and Tanimoto coefficient [5].

After obtaining the potential drug targets, we analyzed the significantly enriched KEGG biological pathways and GO biological processes of these potential targets using the hypergeometric cumulative distribution test [6]. GO annotations of human proteins were obtained from the GO project Web site (http://www.geneontology.org/) [7]. KEGG biological pathway data were downloaded from the KEGG database [8].

0.1% targets were determined in our study mainly because the results were reliable according to reports, including the original paper and our previous study [1, 2]. The reasons why the final 7 pathways were selected were enrichment of both P-value and coverage rate of these pathways were significant.

In fact, we had reported the similar approaches in our previous published paper in 2012 as we cited [2], so we added the brief information in present manuscript to avoid duplication. If it’s necessary, we will describe it in more details.

In the statistical analysis section, ANOVA using SAS 9.2 statistical software (SAS Institute, NC, USA) was applied to evaluate between-group differences in the outcome variables, follow-up least significant differences (LSD) analysis verified these differences were significant (P<0.05).

Reference:


the Coronary Heart Disease,” Evidence-Based Complementary and Alternative Medicine, vol. 2012, Article ID 698531, 10 pages, 2012.


3. Results

a. The search for DQP compounds appears to have been done through the Chinese Materia Medica only, which is far from comprehensive. But, what was the search strategy? Furthermore, how did you verify that the compounds frequently found in Danshen and Sanqi are found in the processed DanQi pill?

Indeed, just as you mentioned, the search for DQP compounds have been done through the Chinese Materia Medica only, which is far from comprehensive. In fact, our results contain most of the acknowledged compounds which were considered as the main active ingredients. As the development of separation and identification technologies, we will update the results in our future study. Moreover, because our formula just contains 2 herbs, so our search strategy is by manual reading to make the results more accurate. We presume all components of herbal formulation compounds are absorbed and utilized. In fact, the distribution and metabolism of herbal formulation in the body are not taken into consideration in our research. In TCM, it’s a new and complicated research area of drug metabolism. Our original purpose is to provide a potential method for pharmacology research on TCM. We have listed your valuable suggestions as the limitation in our revised paper.
b. It is unnecessary (and misleading) to be overly precise reporting your results (eg. EF values … dropped by 49.03%). Also, please report exact p-values rather than NS, p<0.05, p<0.01. What are the actual data values for figure 1?

As you suggested, we reported exact p-values in different groups. And the actual data values for figure 1 were in Table 3, including the results of EF, FS, LVEDs and LVEDd.

c. how was ‘significantly improved’ defined for cardiomyocyte hypertrophy?

There may be an improper use of words “improved”. Here we concluded it as “DQP had the inhibitory effect on ventricular hypertrophy” in our revised paper.

d. Please report absolute values rather than relative values – this should be done throughout the manuscript. Were baseline levels of COX, etc. measured? The appropriate comparisons here should be the amount of change from baseline among groups, which is an analysis that was not described in the section on analysis plan. At the end of paragraph 5, cardioprotective effects are demonstrated clinically, not by estimating metabolite levels.

The baseline levels of COX, etc. in different groups were detected by the westernblot. The forms of the final reported data of westernblot were the normalized band densities of different indicators by GAPDH. As you suggested, we gave the baseline levels in different group. The majority of studies pertaining to endogenous biosynthesis of PGI2 and TXA2 in human disease were invalidated because PGI2 and TXA2 production in the human coronary circulation were undetectable [1]. PGI2 and TXA2 have very short half-life hence their more stable metabolites, 6-keto-PGF1α and TXB2 were often measured instead, and they were applied in many studies in cardiovascular disease [2-3], the 6-keto-PGF1α/TXB2 ratios (P/T) are also always presented in clinical [1], so we speculated DQP might have cardioprotective effects by decreasing these metabolites levels.

Reference:
4. Tables and Figures

a. In general, a table or figure should be able to be read and understood entirely on its own (without having to refer back to the text).

Table 1 – the method for the KEGG analysis should be fully explained in the Methods section (foot note a). The concept of coverage is not entirely clear.

We added it in the section of “2.1 Drug–target prediction and analyses”.

According to the method, the candidate target-genes were firstly predicted by the similarity of herbal compounds structure. Then the candidate target-genes were enriched in different pathway. In each pathway, the percentage of enriched candidate gene among all the genes in this pathway is defined as the coverage rate.

b. Table 2 – method for GO analysis needs to be explained in methods.

We added the content as you suggested.

c. Table 3 – this table would read better with the rat models across the top and the outcome measures vertically. P-values for ANOVA can be clearly reported in a 4th column and t-tests (or whatever was actually used) can also be reported for pair-wise comparisons.

As you suggested, we changed the table and add the P-values.
d. Table 4 – what is ratio of P/T. Please report actual p-values (for both ANOVA and other testing).

Ratio of P/T is the PGF1α to TXB2 (P/T) ratio, as described in “3.2.3 Validation of the predicted target pathways”. And the actual p-values were added.

e. Figure 1 – although the pictographs are nice, actual measurement values would be far more informative.

As mentioned above, the actual data values for figure 1 were in Table 3, including the results of EF, FS, LVEDs and LVEDd.

f. Figure 3 – why are there 2 asterixes over the ‘model’ group?

There may be some reasons as the commercial antibody had Non-specific staining, or the exposure time we took was too long, but the target band was in the right region of 75kD. And the “2 asterixes” were not analyzed during the final statistics. We’ll improve our experimental procedure. And we can see the similar western blot results of COX2 in other article [1].


g. Manuscript title – ‘balancing’ and ‘patterns’ are vague descriptions. Furthermore, these studies did not look at the protective effects of DQP against HF development. In a rodent HF model, you demonstrated that DQP could reverse protein expression that is induced in the HF model.

We revised the descriptions to make them more accurate. As we described in the manuscript, an increase in left ventricular collagen (cardiac fibrosis) is a detrimental process that adversely affects heart function, and it’s also an important aspect of heart failure (HF) and an index of poor prognosis [1]. Strong evidence implicates the infiltration of inflammatory cells as a critical part of the process resulting in cardiac fibrosis. Inflammatory cells are capable of releasing arachidonic acid (AA), which may be further metabolized by cyclooxygenase and lipoxygenase to biologically
active products, including PGs, leukotrienes, etc \[2\]. Some of these products have profibrotic properties and may represent a pathway by which inflammatory cells initiate and mediate the development of cardiac fibrosis. Some drugs are produced to target on the pathway. In our study, the DQP can decrease the LVEDd and LVEDs, and it also can inhibit the AA metabolism. So we assumed that the AA metabolism is the potential target for DQP to inhibit the cardiac fibrosis.

Reference:


We have also improved our languages in new manuscript, and some new references were added.

Thank you for the important suggestions.

We hope that the revised manuscript could satisfy the requirements for publication of BMC. Thank you and the reviewers again for your help.

Sincerely yours,

Wang Wei

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