Author's response to reviews

Title: Spironolactone ameliorates endothelial dysfunction through inhibition of the AGE/RAGE axis in a chronic renal failure mouse modelRunning title: Mineralocorticoid receptor antagonist, advanced glycation end products, chronic renal failure

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Spironolactone ameliorates endothelial dysfunction through inhibition of the AGE/RAGE axis in a chronic renal failure mouse model

Running title: Mineralocorticoid receptor antagonist, advanced glycation end products, chronic renal failure.

Chun-Cheng Wang; An-Sheng Lee; Shu-Hui Liu; Kuan-Cheng Chang; Ming-Yi Shen; Chiz-Tzung Chang

BMC Nephrology

Dear Dr. Chang,

Your manuscript "Spironolactone ameliorates endothelial dysfunction through inhibition of the AGE/RAGE axis in a chronic renal failure mouse model

Running title: Mineralocorticoid receptor antagonist, advanced glycation end products, chronic renal failure."

(BNEP-D-19-00255) has been assessed by our reviewers. Based on these reports, and my own assessment as Editor, I am pleased to inform you that it is potentially acceptable for publication in BMC Nephrology, once you have carried out some essential revisions suggested by our reviewers.

Their reports, together with any other comments, are below. Please also take a moment to check our website at

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We request that a point-by-point response letter accompanies your revised manuscript. This letter must provide a detailed response to each reviewer/editorial point raised, describing what amendments have been made to the manuscript text and where these can be found (e.g. Methods

section, line 12, page 5). If you disagree with any comments raised, please provide a detailed rebuttal to help explain and justify your decision.

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We look forward to receiving your revised manuscript and please do not hesitate to contact us if you have any questions.

Best wishes,

Xiong-Zhong Ruan

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Technical Comments:

Editor Comments:

The study is well-designed and clearly report. However, the reviewers have pointed out several concerns.

The main problems are that the quality of data should be improved and the statistical analysis should be redone using the proper method.

BMC Nephrology operates a policy of open peer review, which means that you will be able to see the names of the reviewers who provided the reports via the online peer review system. We encourage you to also view the reports there, via the action links on the left-hand side of the page, to see the names of the reviewers.

Reviewer reports:

Jesper Nørgaard Bech, MD, PhD (Reviewer 1): The study by Wang et al reports the effects of in vivo treatment with spironolactone or AGE-breaker (ALT-711) in a rat model of non-diabetic CKD on renal function, vascular reactivity (endothelial- and non-endothelial dependent). From the in vivo model, immunohistochemistry studies were performed in vascular tissue, ie. distribution of eNOS, P-eNOS and anti-RAGE. In parallel, a range of in vitro studies were performed on HAEC's to elucidate on the potential mechanisms of action of spironolactone with emphasis on the interaction with the AGE/RAGE axis. In these studies, the authors used cell viability assay, Western blots and confocal microscopy.

General comments

The study is well designed and clearly reported. The authors are able to demonstrate significant effects of spironolactone in vivo on AGE-related effects and effects on vascular reactivity. The extension of these observations to cell-derived studies using HAEC's supports the notion, that important aspects of the clinical effects of spironolactone perhaps could be explained by the proposed upregulation of SIRT3 and NOX.

Specific comments

In the title and a few places in the text (p. 23, line 15), the authors refer to "a mouse model".....the study was performed on Sprague-Dawley rats.

Thanks for your comment, we changed “ a mouse model” into “rat model”

The authors suggest, that there was no difference in the in vivo effects of spironolactone vs. ALT-711 on renal function based on analysis of the BUN-results. However, looking at the creatinine data (a better estimate of GFR...?) suggests, that creatinine levels rose by approx. 107 % in Groups 2 and 3, but only 22 % in Group 4 (spironolactone). This seems to be a quite significant difference and may suggest a differential effect of ALT-711 vs. spironolactone on renal function. This should be clarified by the authors, i.e. whether this represents a power issue ? In line with this - the levels of BUN could reflect other issues than renal function (protein intake, diuresis etc...)

Thanks for your comments!!

We compared the plasma Creatinine data between Group 2 (CKD group), Group 3 (CKD+ ALT-711 group) and

Group 4 (CKD+ Spironolactone) with ANOVA and post-hoc comparison with LSD, we did not notice a significant difference regarding Creatinine level between Group 2 vs. Group 3, and Group 2 vs. Group 4. Due to limited case numbers, we cannot exclude the possibility of power issue. More case numbers may support previous observations that treatment with ALT-711, or spironolactone could improve renal function. Due to different renal function decline rate, we cannot exclude the possibility that ALT-711 and spironolactone have differential （彳亍 effects and mechanism. It is generally known that AGEs may interact with RAGEs, LOX,… thereby induce inflammatory effect, while spironolactone may interact with mineralocorticoid receptor, androgen receptor, estrogen, progesterone receptor, glucocorticoid receptor. Therefore, the two drugs may both improve endothelial dysfunction through different and myriad pathways. Our study does not suggest that ALT-711 and spironolactone both improve endothelial dysfunction through same mechanism. In our study, we tried to provide an evidence that spironolactone may improve endothelial dysfunction through inhibition of AGEs/RAGE axis.

The inhibition of AGEs/RAGE axis may only be one of the many ways by which spironolactone acts to improve endothelial dysfunction. We thanks for reviewer’s invaluable advise: (1) Based on observation of differential renal function decline, the spironolactone and AGEs may have

different effect and mechanism on renal function improvement, which may further contribute to improvement of endothelial dysfunction. (2) The high BUN/Crea level in the Group 4 after treatment in comparison with those of the other three groups may be

Explained by the inherent diuretic effect of spironolactone. (Given by same chow diets in four group, we thought

The high BUN/Crea level in Group 4 may not be explained by high protein intake)

We have added this point into Discussion and study limitation!!

Please see P. 21 and P. 24

P. 21

"Though our study failed to demonstrate significant differences of plasma creatinine levels after the treatment between

Groups 2,3 and 4. A lower renal function decline rate could be observed in Group 4 in comparison with those of Group 2 and 3. This indicates that spironolactone and ALT-711 may have differential effects and mechanism on renal function.

Our study cannot exclude the possibility that by application of other renal failure models, spironolactone and

ALT-711 may improve endothelial dysfunction through improving renal function by different pathways."

P. 24

Fourth, compared with Group 2, we failed to demonstrate significantly lower plasma creatinine level in Group 4. This implied that spironolactone failed to improve renal function in 5/6 radial nephrectomy renal failure model. However, due to limited numbers with inadequate statistical power, the interpretation of the study result should be cautious. In addition, numerically higher BUN/Creatinine ratio after the treatment in Group 4 was noted. This indicated that the renal protection effect of spironolactone may be further confounded by its diuretic effect. [32,33]

The in vivo study might improve by reporting blood pressure data (those were measured according to the methods section) and body weights of the animals

Thanks for your comments!! We have added body weights and systolic blood pressure data in Table 1

Why did the authors not include a group of animals subjected to combination treatment (ALT 711 + spironolactone)? This would have provided more information about the in vivo importance of the suggested pathways.

Thanks for your comments!! In our study aim and design, we investigate whether spironolactone could improve endothelial dysfunction through inhibiting AGEs/RAGE pathway. Therefore, we used CKD + ALT-711 (AGEsbreaker) as a control group. We demonstrated that similar pattern of IHC results, vasoreactivity results, plasma

AGEs levels results could be observed in CKD + ALT-711, and CKD + Spironolactone groups. Thus, we further conduct cell-derived experiments to investigate whether spironolactone could upregulate the expression of RAGEs (which could positively enhance the AGEs/RAGE axis cascade). We aim to investigate whether spironolactone could inhibit or counterregulate the activation of AGEs/RAGE axis, and we did not plan to discuss the interaction between spironolactone and ALT-711 (an AGEs breaker). As a result, we did not design a group, of which CKD rats were treated with both ALT-711 and spironolactone.

Sirirat Anutrakulchai, M.D.,PhD (Reviewer 2): The authors studied the effect of spironolactone on reduction of endothelial dysfunction in Sprague-Dawley rats which developed CKD with 5/6 nephrectomy model.

Results demonstrated the benefit of treatment with possible mechanisms. The authors have performed the intensive study and I just have some minor comments.

1. Sprague-Dawley are rats, not mice, therefore the title of mouse model is not quite true.

Thanks for your comment, we have revised our title as follows:

"Spironolactone ameliorates endothelial dysfunction through inhibition of the AGE/RAGE axis in a chronic renal failure rat model"

2. In "Statistical analysis", comparison between before and after treatments in the same group should use

Paired T test.

Thanks for your comment!!

After we re-do the statistics with paired student t-test for comparisons of BUN, Crea levels before vs. after

Treatment (Table 1), and plasma AGEs levels before vs. after treatment (Figure 2A). The result remains same as previous analysis.

We have revised our statistical analysis and Abstract "material and method section" as follows:

P3

"To investigate the underlying mechanism, we cultured human aortic endothelial cells (HAECs) for in-vitro assays.

Differences between two groups were determined with the paired student's t test."

P14.

Statistical analysis

Continuous variables are expressed as mean ± standard error from at least three experiments. Differences between two groups were determined with the paired student's t test.

3. The line 18 of page 17, "is aer" should be corrected.

Thanks for your comment, we have corrected it!!

"To evaluate whether spironolactone has an inhibitory effect on the AGEs/RAGE axis, we pre-treated the

AGEs-stimulated HAECs with spironolactone at different concentrations (0.1, 1, and 10μM) and the results is are presented in Figure 5."

Aihua Zhang, Ph.D., M.D. (Reviewer 3): This research showed the protective role of Spironolactone in ameliorating endothelial dysfunction in a 5/6 nephrectomy renal failure model though inhibition of the AGEs/RAGE axis, upregulation of SIRT3, and attenuation of NOX-2 associated intracellular oxidative stress.

This study is in line with some similar reports on the cardiovascular benefits effect of Spironolactone in heartfailure, diet-induced obesity, and a streptozocin-induced diabetic model. The experiments are well designed.

However, I still have the flowing comments.

1. In this study, the author applied the 5/6 nephrectomy renal failure rat model. Did the authors measure the blood pressure? Whether Spironolactone affects the hypertension caused by 5/6 nephrectomy renal failure.

Thanks for the comments!! We have added the blood pressure data and body weight data as requested by reviewers and displayed in Table 1. We did not find significant differences between CKD group (Group

2) and CKD + Spironolactone group (Group 4).

2. The resolution of the graphs is poor. eNOS and p-eNOS are mainly expression in endothelial cells, it is very hard to observe the signal in figure 3B and additional file1.

Thanks for the comments!! We are very sorry for the ignorance of our image quality, which was only 96d.p.i. We have improved the image resolution to 600d.p.i. in the revised figures (Figure 3, and Additional file 1)

3. The cell viability of HAECs treated with BSA and AGEs were measured by MTT assay in additional file2.

The results showed that AGEs or BSAs at a concentration of 500μg/dL for 24 hours was toxicity to HAECs. Why author still used the concentration in Figure 4.

Thanks for the comments!! In our study, we just tried to establish a concentration-dependent effect of AGEs-

Induced RAGE expression and phosphorylation of eNOS. In view of our MTT data, we later chose to use AGEs

200ug/ml for the following experiments to survey the detailed mechanism involved.

4. Why is there no error bar in the vehicle group in Figure 4 and Figure 4. It would be nonsensical to compare any groups with the vehicle group.

Thanks for the comment!! In our study, we compare and adjust each data with its own vehicle group. We thought it may somehow reflect the upregulation or down regulation of the target protein of interest in each experiment. As a result, for each vehicle group, it represents 1, and there's no error bar in the vehicle group.

Similar method has been conducted and published in other literatures.

Ref. 1. Lee SD, Chu CH, Huang EJ, Lu MC, Liu JY, Liu CJ, et al. Roles of insulin-like factor II in cardiomyoblast

Apoptosis and in hypertensive rat heart with abdominal aorta ligation. Am J Physiol endocrinol Metab. 2006.

291;E306-E314.

Ref 2. Wu Y, Li Y, Zhang C, Wang Y, Cui W, Li H, Du J. S100a8/a9 released by CD11b+Gr1+ Neutrophils

Activates cardiac fibroblasts to initiate angiotensin II-induced cardiac inflammation and injury. Hypertension.

2014;63:1241-1250.

5. It is necessary to detect the toxicity of Spironolactone in HAECs.

Thanks for the comment!! We have conducted the MTT study of spironolactone, and the result is depicted in

Additional file 2C. No specific drug toxicity of spironolactone at concentrations from 0.1-10μM was noted.

6. The western blot bands of SIRT3 and p-eNOS in Figure 7 were not representative. It is hard to observe the expression of SIRT3 and p-eNOS in AGE treated group is less than vehicle group.

Thanks for the comment!! In our study, we conducted 3 separate experiments for each figure. As a result, the quantified results are the average of the 3 separate experiments analyzed by image J. The affiliated Excel file isthe raw datas of Figure 7.

Figure 7(A) raw data

First data	GAPDH	SIRT3 (1)	SIRT3/GAPDH	SIRT3/GAPDH
Vehicle	17231.67	18556.57	1.076888	1
BSA 200	20707.69	15009.42	0.724823	0.673073
AGE 200	20483.88	11342.82	0.553744	0.514208
AGE 200ug/dL + RAGE Aby	21305.88	17934.83	0.841778	0.781677
AGE 200 + SPL 0.1	23228.93	6576.004	0.283095	0.262883
AGE 200 + SPL 1	19968.94	13009.05	0.651464	0.604951

AGE 200 + SPL 10	19643.64	11055.86	0.562821	0.522637

Second data	GAPDH	SIRT3 (2)	SIRT3/GAPDH	SIRT3/GAPDH
Vehicle	15403.52	16965.86	1.101428	1
BSA 200	19721.69	13162.76	0.667426	0.605964
AGE 200	22716.13	10970.18	0.482925	0.438453
AGE 200ug/dL + RAGE Aby	19203.05	22298.3	1.161185	1.054254
AGE 200 + SPL 0.1	22212.88	10660.35	0.479917	0.435723
AGE 200 + SPL 1	19915.76	15937.81	0.800261	0.726567
AGE 200 + SPL 10	14988.45	10912.05	0.728031	0.660988

Third data	GAPDH	SIRT3 (3)	SIRT3/GAPDH	SIRT3/GAPDH
Vehicle	21298.28	21369.23	1.003331	1
BSA 200	25377.64	19801.3	0.780265	0.777675
AGE 200	27827.3	16702.06	0.600204	0.598212
AGE 200ug/dL + RAGE Aby	25029.52	27378.71	1.093857	1.090225
AGE 200 + SPL 0.1	26184.76	18024.47	0.688357	0.686072
AGE 200 + SPL 1	25429.05	20738.3	0.815535	0.812828
AGE 200 + SPL 10	21097.28	17578.88	0.83323	0.830463

Figure 7(B) raw data

First	p-eNOS	eNOS	GAPDH	p-eNOS/eNOS	eNOS/GAPDH		
Vehicle	14893.18	20388.83	20665.57	0.730457	1	0.986609	1
AGEs 200ug/mL	13567.28	20190.95	26609.76	0.671948	0.919901	0.75878	0.769079

AGEs 200ug/mL + Neutralizing RAGE Aby	20399.37	22403.95	26117.88	0.910525	1.246514	0.857801	0.869444
AGEs 200ug/mL + SPL 10uM	22324.9	26754.78	26365.05	0.834427	1.142334	1.014782	1.028556
AGEs 200ug/mL + SPL 10uM + Tenolvin-6 0.1uM	23821.27	23981.49	23804.76	0.993319	1.359859	1.007424	1.021098
AGEs 200ug/mL + SPL 10uM + Tenolvin-6 1uM	13363.86	18330.13	19996.64	0.729065	0.998094	0.91666	0.929102

Second	p-eNOS	eNOS	GAPDH	p-eNOS/eNOS	eNOS/GAPDH		
Vehicle	9454.933	12258.93	17200.35	0.771269	1	0.712714	1
AGEs 200ug/mL	11886.05	16987.61	20150.62	0.69969	0.907193	0.843032	1.182847
AGEs 200ug/mL + Neutralizing RAGE Aby	21268.42	22881.9	23162.95	0.929486	1.205139	0.987866	1.386063
AGEs 200ug/mL + SPL 10uM	22304.37	21965.49	24597.2	1.015428	1.316568	0.893008	1.252968
AGEs 200ug/mL + SPL 10uM + Tenolvin-6 0.1uM	12455.32	18147.56	25565.2	0.686336	0.889878	0.709854	0.995987
AGEs 200ug/mL + SPL 10uM + Tenolvin-6 1uM	10124.81	16628.35	25223.83	0.608888	0.789463	0.659232	0.92496

Third	p-eNOS	eNOS	GAPDH	p-eNOS/eNOS	eNOS/GAPDH		
Vehicle	17245.18	20425.3	21111.15	0.844305	1	0.967512	1
AGEs 200ug/mL	13530.28	20009.13	26775.35	0.676205	0.800902	0.747297	0.77239
AGEs 200ug/mL + Neutralizing RAGE Aby	20158.37	23156.25	26150.18	0.870537	1.031069	0.88551	0.915245

AGEs 200ug/mL + SPL 10uM	21827.95	25835.08	26485.93	0.844896	1.0007 0.975426 1.00818
AGEs 200ug/mL + SPL 10uM + Tenolvin-6 0.1uM	23731.56	24622.95	24310.64	0.963798	1.141529 1.012847 1.046857
AGEs 200ug/mL + SPL 10uM + Tenolvin-6 1uM	14139.28	19150.18	20619.69	0.738337	0.874491 0.928732 0.959918

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- Ethics approval and consent to participate

- Consent to publish

- Availability of data and materials

- Competing interests

- Funding

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