Author’s response to reviews

**Title:** Indoxyl sulfate, a uremic toxin, downregulates renal expression of Nrf2 through activation of NF-kappaB

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**Author’s response to reviews:** see over
Dear Editor,

I submit a revised manuscript for publication in BMC Nephrology entitled, “Indoxyl sulfate, a uremic toxin, downregulates renal expression of Nrf2 through activation of NF-κB”.
I revised the paper according to the reviewers’ comments.

We first demonstrated that indoxyl sulfate downregulated the expression of Nrf2 in human proximal tubular cells through activation of NF-κB. Furthermore, indoxyl sulfate downregulated expression of Nrf2 in rat kidneys, and AST-120, an oral sorbent which reduces serum level of indoxyl sulfate, upregulated expression of Nrf2 in the kidneys of CKD rats.
Taken together, indoxyl sulfate downregulates Nrf2 expression through activation of NF-κB in the kidney, followed by downregulation of HO-1 and NQO1 and increase of ROS.

I believe that this paper will stimulate further research on the uremic toxicity of indoxyl sulfate.

I would be pleased if you consider its publication in BMC Nephrology.

Sincerely yours,

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Responses to reviewers' comments

I sincerely appreciate the valuable comments of the reviewers.

Reviewer 1

1. In the results, it should be clarified whether the western blots are whole cell or tissue lysates or nuclear fractions. If they were whole cell lysates, why were nuclear fractions not investigated. The subcellular location of Nrf2 could be very informative in regards to Nrf2 regulation in this model.

Page 12, lines 8-10
Because the expression of Nrf2 protein in the whole cell lysates was reduced, the expressions of its downstream genes are considered to be decreased.

2. It would be easier to review the serum levels of indoxyl sulfate in tabular form instead of simply reporting in the text.

I added Table 1 listing serum levels of indoxyl sulfate in the revised paper.

3. Please clarify the subheadings within the results section with bolded or italicized text.

The subheadings were clarified with bolded text.

4. There are better Nrf2 target genes than HO-1. The article would be greatly improved with the addition of others, such as Nqo1, sulfiredoxin 1, etc.

I included immunohistochemical data of NQO1 in Fig. 4-7, and in the text.

5. The article would be improved with the addition of NFkB targets.

Page 16, lines 4-6 from the bottom
Because we focused on the crosstalk between indoxyl sulfate and Nrf2, expressions of the NF-κB targets such as Keap1 and p53 are not included in the present study,
6. The article would benefit greatly with grammatical revisions.

I improved the paper grammatically including the specific grammatical revisions as suggested by the reviewer.

Reviewer 2
Major Compulsory Revisions,
1. For in vivo study, the authors used two animal models: spontaneous hypertension rat model and 4/5-nephrectomy CKD rat model. Why do you choose different animal models for in vivo study? Appropriate reasons should be given.

Page 15, the last paragraph
The present study used two animal models. In animal study 1, the effects of indoxyl sulfate administration on Nrf2 expression in the kidneys of not only normal rats but also hypertensive rats were determined, because hypertension is often associated with CKD. In animal study 2, the expression of Nrf2 in the kidney of CKD rats was evaluated as compared with normal rats. Furthermore, the effect of AST-120 on Nrf2 expression in the kidneys of CKD rats was determined, because AST-120 reduces serum indoxyl sulfate level and is clinically used to treat CKD patients in Japan.

2. The quality of immunohistochemical staining (Fig.4 and Fig.6) was poor. Please change these photos.

I changed the photos in Fig. 4 and Fig. 6 in the revised paper.

Minor Essential Revisions,
1. “…… HO-1, a downstream gene of Nrf2 and an antioxidant gene, ……” this sentence has been repeated several times in this article.

The sentence was not repeated in the revised paper.

2. Abbreviations should be used appropriately.
(1) Page 2. In abstract, “HK-2 cells” should be used as “human proximal tubular cells” comes out the first time.

Page 2, lines 11-12
HK-2 cells as human proximal tubular cells

(2) Page 5. For introduction part, “epithelial-mesenchymal transition (EMT)” is no need to be used, because this term was used only one time in this article.

Page 6, line 7
The word “(EMT)” was deleted.

(3) Page 7. “Human proximal tubular cells” should be given together with “HK-2 cells”.

Page 8, line 2
HK-2 cells (human proximal tubular cells)

3. Page 2, In abstract, “Nrf2 and its related genes is downregulated.” Here “is” should be changed with “are”.

Page 2, line 5
(Nrf2) and its related genes are

4. Page 5, “The expression of Nrf2 and its related genes is impaired in kidneys of CKD rats [20].” Here “is” should be changed with “are”.

Page 6, line 5 from the bottom
its downstream target genes such as HO-1 and NQO1 are

5. Page 7. The concentration of insulin-transferrin-selenium should be given.

Page 8, lines 3-4
insulin (10 µg/ml insulin-transferrin (5.5 µg/ml)-selenium (6.7 ng/ml),

6. Page 8. The authors mentioned “indoxyl sulfate (200mg/kg of indoxyl sulfate
in water)”, is this dose (200mg/kg) for one day?

Page 9, line 3 from the bottom

(200 mg/kg/day of indoxyl sulfate in water)