Author's response to reviews

Title: Impaired endocytosis in proximal tubule from subchronic exposure to cadmium involves angiotensin II type 1 and cubilin receptors.

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Author’s response to reviews: see over
August 24th, 2013

Dr. Hayley Henderson
Executive Editor
BMC Nephrology

Dear Dr. Henderson,

Enclosed please find the revised version of our manuscript entitled "Impaired endocytosis in proximal tubule by subchronic exposure to cadmium involves angiotensin II type 1 receptor." by Santoyo-Sanchez et al.

Title has been modified to better express the new findings obtained after performing the experiments requested by reviewer 1: “Impaired endocytosis in proximal tubule from subchronic exposure to cadmium involves angiotensin II type 1 and cubilin receptors”.

I would like to thank you for the valuable comments regarding to the manuscript. All comments and suggestions made for the reviewers were considered as a result most of some sections of the manuscript were modified and strongly improve our manuscript considerably. All the changes made appear in a red font in the new version of the manuscript.

We have carefully followed the recommendations of the journal and we declare no conflict of interest.

All other Authors have read the manuscript and have agreed to submit it in its current form for consideration for publication in BMC Nephrology.

Looking forward hearing from you, I remain.

Yours faithfully,

Dr. Olivier Christophe Barbier
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Reviewer 1: Dr. Isabelle Rubera
Thank you very much to the reviewer for her positive comments that have improved our manuscript.

Major Compulsory Revisions
-Intoxication protocol / cadmium accumulation in the kidney / kidney damage.

1) It would be interesting to compare and discuss the results of this study with others, concerning the route of administration (oral), the duration of treatment and the Cd content in the kidney. In the literature, subcutaneous or intraperitoneal injections of lowest dose of Cd have been shown to induce highest Cd concentration in kidney and kidney failure. Likewise, how an equivalent amount of Cd (around 5-7 µg/g) in the kidney can induce different kidney damage phenotype? (for example, Jacquillet et al., Am J Physiol Renal Physiol. 2006 Jan;290(1):F127-37)

Oral absorption of Cd is about 10% and it is distributed to different target organs, thus, oral route shows a low accumulation and uptake of cadmium; meanwhile in intraperitoneal administration, this metal enters to the peritoneal cavity and all metal diffuses almost immediately to the blood and then it is distributed to different target organs (Liver and Kidney). For these reason, intraperitoneal route shows quick/high uptake and accumulation. Subcutaneous administration released gradually the cadmium. It has been observed that accumulation and uptake of Cd, is different in both genders, routes of administration and exposure time to Cd (Nowkocha et al. Niger J Physiol Sci 2011, 26(1):97-101; Hofer et al Toxicol Lett 2009, 191(2-3):123-131).

In our model, the administrated dose of Cd (3mg/kg/day) for 8 weeks induced Cd content of 7.6 µg/g kidney. We only determinate Cd content at 8 weeks, we do not know the tissue concentration before 8 weeks, for these reason, we do not know how long the tubule proximal cells were exposed to these concentrations of Cd. Concerning the work of Jacquillet et al. 2006, the dose of Cd used was 500µg/kg/day by intraperitoneal injection during 5 days (sub-acute exposure). They observed that Cd content gradually increased after each administration, and reaching a maximum level which was maintained until day 20. They evaluated glomerular and tubular function, using different markers of damage as inulin clearance and fractional excretions of different ions, and the major effects was observed around day 10. We only determine the fractional excretion of Na+ at 8 weeks (data not shown) and no differences compared to the control group were evidenced.

To improve the understanding of the manuscript, we decided to rewrite this part of the discussion. The new version is this one (page 18 line 11):

‘In this study, we decided to administer Cd by gavage, for two main reasons: 1) to control the administered dose, and, 2) oral route reflects the dietary exposure that is the most frequent route in the non-smoker population [4-6]. To better assess cellular mechanisms involved in Cd-nephrotoxicity, we
decided to use a low dose that would not induce extent damage in proximal tubules.”

2) Microalbuminuria is the only renal parameter tested affected in those Cd-treated animals. The authors may provide additional studies and test an early biomarker of Cd-induced proximal tubule injury such as Kim-1.

To improve our results, we performed the analysis of Kim-1 expression using fluorescence microscopy with the antibody polyclonal goat anti Kim-1 antibody (Cat. No. AF3689, R&D System, dilution 1:500) using dipeptidyl-peptidase (IV)2 (MCA924, Serotec Ltd., Kidlington, Oxford, OK, dilution 1:500) as a marker of proximal tubules.
Our results show that if classic biomarkers for proximal damage (Urinary glucose and NAG) did not show changes, Kim-1 as a specific and early biomarker of tubular damage, increased after cadmium-exposure and was not present when Losartan was co-administrated.

The results observed with Kim-1 are consistent with those observed with microalbuminuria.

This new data was added in the revised version of the manuscript as a new figure (Fig 3), in methods, results and discussion sections.

Thank you for your comment that improves the revised manuscript.

-oxidative stress parameters.
3) In the Table 2, lipid peroxidation index is diminished in LOS-treated and Cd-treated groups. The authors need to explain.

Indeed, lipid peroxidation seems to decrease in LOS and Cd groups. Nevertheless, this trend was not significant using ANOVA test comparing all groups or using STUDENT test comparing each group with the control.

Considering the possible lack of sensitivity of the MDA technique in our experimental design, we attempted to evaluate Nuclear factor-erythroid-2-related factor 2 (Nrf2) by western-blot technique. This transcription factor binds to antioxidant response elements and regulates the expression of antioxidant genes. Results show that Nrf2 increased in LOS, Cd and LOS+Cd groups compared with Control, suggesting that each treatment induced a cellular response to oxidative stress, intending to increase the anti-oxidant response (fact that we were not able to evidence using MDA determination).

Unfortunately, we only have total protein extracts, in order to be sure, we need to evaluated Nrf2 in nuclear extract. But we don’t have more samples and only could perform a unique experiment. For this reason, we show you the result of the WB but cannot increase the number of experiment for the moment.
Thank you for your comment that improves the revised manuscript.

To improve the understanding of the manuscript, we decided to rewrite this part of the discussion. The new version is this one (page 22 line 10):

“In our model, we did not find an increase in oxidative stress, probably due to an antioxidant response of cells.”

4) Upregulation of the expression of pro-oxidant enzyme NADPH oxidase has been reported in several models of Cd intoxication. The authors may enrich the discussion to explain how NADPH activity is inhibited in Cd+LOS group and only in this group.

Page 20 line 19. What is the reason to cite Reference 48 (Renugadevi et al., Toxicology. 2009 Feb 4; 256(1-2):128-34) here in the discussion as no NADPH oxidase activity has been studied in this paper.

We found only one article where low Cd concentration decrease NADPH activity, and it is known that AngII increases NADPH oxidase activity through AT1 receptor, thus LOS (an AT1 antagonist) decrease NADPH activity. In our result, we suggest a synergistic effect by observing a statistically significant decrease in Cd+LOS group.

We apologize for cite (Renugadevi et al., Toxicology. 2009 Feb 4; 256(1-2):128-34). The reference has been removed.

The new version is this one (page 22 line 4):

“Thijssen et al. (2007) report that Cd exposure may trigger a biphasic defense response in the kidney, and could lead to adaptation and survival [55] maybe by induction of Nuclear factor erythroid 2 related factor 2 (Nrf2), this transcription factor binds to antioxidant response elements and regulates the expression of antioxidant genes [56]. On the other hand, and it has been suggested that Cd at low concentrations could modulate and inhibit NAD(P)H oxidase activity [57].”

-megalin expression/ tubular endocytosis.

5) The megalin-independent Cd-induced microalbuminuria is an interesting observation but it need to be followed up and the involvement of cubulin has to be tested in the current study.

To improve our results, we performed the analysis of Cubilin expression using fluorescence microscopy with the antibody polyclonal goat anti-cubilin antibody
(sc-20609, Santa Cruz Biotechnology, Inc., dilution 1:50), using monoclonal mouse anti-dipeptidyl-peptidase (IV) 
2 antibody (MCA924, Serotec Ltd., Kidlington, Oxford, OK, dilution 1:500 ) as a marker of proximal tubules. This new data was added in the revised version of the manuscript as a new figure (Fig 5), in methods, results and discussion sections. Cubilin protein expression was altered by Cd treatment, whereas co-administration of LOS (Cd+LOS group) maintained Cubilin levels, suggesting that cubilin is the responsible of microalbuminuria and that AT1 and cubulin are related.

Thank you for your comment that strongly improves the revised manuscript

6) Legend of Figure 3 and description of Figure 3 in Results section correspond to Figure 4 and vice versa
Thank you for the comment and we apologize for the original oblivion. Error was corrected (page 29).

**Minor Essential Revisions:**
-Page 18 line 16: correct the sentence “however, receptor-mediated endocytosis was affected as evidenced by the presence of microalbuminuria” suggested instead of evidenced; no experiments have been performed for receptor-mediated albumin endocytosis
This sentence was deleted, because the new result showed a possible relationship between cubilin receptor and microalbuminuria induced by cadmium exposure.

-Page 18, latest line: define MT1/2
MT1/2 was defined “Metallothionein 1/2”.

-Page 20, latest line: correct “we used a Losartan (AT1 receptor antagonist);”
This sentence was corrected “we used Losartan, an AT1 receptor antagonist...”

-Page 21 line 2: other studies instead of others studies
This sentence was corrected (page 22 line 19).

-the authors may add and discuss a new paper recently published “Effect of angiotensin II type 1 receptor blocker on renal function, arterial blood pressure and parathyroid hormone related protein over expression in cadmium induced nephrotoxicity in adult male rats” Int J Physiol Pathophysiol Pharmacol 2013;5(2):109-119 by Marwa A Ahmed.
We read carefully this paper by Ahmed during the elaboration of our manuscript, considering with interest the effect of Telmisartan on cadmium-induced nephrotoxicity. However, at the view of cadmium content in kidney in control group (17.58 +/- 1.49 microg/g tissue), we decided to not mention this study because of the possibility that “white male albino rats” (Wistar or Sprague-Dawley?) have
been chronically exposed to cadmium during their whole life or during several generations.
Just to remember: our control group shows 0.08+/−0.00 microg/g wet tissue.
If the reviewer considers that such level of cadmium in control group is not relevant in the study of Ahmed, we will discuss this point in our manuscript. Thank you for the comment.

Reviewer 2: Dr. Mara Medeiros
Thank you very much to the reviewer for her positive comments that have improved our manuscript.

*Discretionary Revisions*
*Please revise English grammar*
We apologized, English grammar of the manuscript was revised by Dr. Andrea De Vizcaya (PhD, University of Surrey, Guildford, England) in this new version of the manuscript.

*Minor Essential Revisions*
Background, page 4 line 10
“Smoking a pack of cigarettes per day is leads to” please delete is
This sentence was deleted from the introduction. Thank you for your observation.

*Major Compulsory Revisions*
*Please add an explanation about the low cadmium dose administered*
To improve the understanding of the manuscript, we decided to rewrite this part of the discussion. The new version is this one (page 18 line 11):
“In this study, we decided to administrate Cd by gavage, for two main reasons: 1) to control the administered dose, and 2) oral route reflects the dietary exposure that is the most frequent route in the non-smoker population [4-6]. To better assess cellular mechanisms involved in Cd-nephrotoxicity, we decided to use a low dose that would not induce extent damage in proximal tubules.”

Please specify if the Cd+LOS group received the losartan treatment during eight weeks or if they started losartan on the fifth week as LOS group. The information was added in page 7 line 13.
“Cd: rats were administered with cadmium chloride (CAS No. 10108-64-2, Sigma Aldrich) (3 mg/kg/day, by gavage) for 8 weeks (weeks 0-8) 4) Cd+LOS: rats were administered with Cd for 8 weeks and LOS from weeks 5-8.”
Thank you for the comment and we apologize for the original oblivion.