Author's response to reviews

Title: Effects of uremic solutes on reactive oxygen species in vitro model systems in monitoring the renal function

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Author's response to reviews: see over
Answer to Reviewers

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The suggestions and questions raised by the reviewers were all pertinent and we agree entirely with their observations. We have made several modifications in the original manuscript to comply with their requests.

After the consideration of the reviewers’ suggestions, we have provided a better way to present the information in the Abstract, Introduction, Results (Table 4) and Discussion, improving the interpretation of our findings.

The reviewers’ requests are highlighted in “red” in the new version of the manuscript, however another changes were made throughout the manuscript attempting to make the text in an intelligible way, including an extensive revision of the English grammar.

We have state the reviewers’ suggestions separately, and each suggestion/question was followed by the corresponding answer, as follows:

- **Reviewer 1:** Suggestions 1 to 38
- **Reviewer 2:** Suggestions 1 to 4
- **Reviewer 3:** Questions 1 to 3

Sincerely,

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Reviewer 1 (Marcela Hermann)

In this work Pires de Assis et al. describe the effect of uremic solutes on reactive oxygen species in vitro model systems. The authors used several uremic solutes that accumulated in blood from patients with chronic kidney disease for test whose antioxidant potential and used several assay to measure antioxidant activities of selected uremic solutes. However the manuscript is not suitable for publication in its current state. Several major issues need to be addressed before the manuscript can be accepted. Many parts of this manuscript are written in a confusing and / or too colloquial manner.

Major Compulsory Revisions

Suggestion 1

According instructions for authors and general guidelines of the journal’s style, following change in sections order is necessary: Methods - Results and Discussion - Conclusion. Do not use numbering of /in sections.

Answer:

The manuscript was changed according to the template of “BMC-series medical journals - authors' checklist for manuscript formatting”, which was sent together with the reviewer’s suggestions (http://www.biomedcentral.com/authors/medicine_journals). The actual order of the manuscript is: Abstract – Background – Methods – Results – Discussion – Conclusion.

Suggestion 2

Change sections name “Materials and Methods” to “Methods” only (P 14, L338)

Answer:

The cited section name was changed (please see P 6, L 121).

Suggestion 3

Subsection 4.1 in manuscript (P 14, L 340) named “Reagents and Chemicals” should be removed, and the information in this section should be given in methods.

Answer:

The subsection “Reagents and Chemicals” was removed from “Methods” section and the related information was distributed throughout the “Methods”.
Suggestion 4

The authors should check references style; in the manuscript the references are not in journal style and not consistently (different font and size e.g. P25. L614 and L 617). See examples for that in section of the References in the BMC Nephrology guide for authors.

Answer:

The reference style was changed according to the template of “BMC-series medical journals - authors' checklist for manuscript formatting”.

Suggestion 5

Statement in the title, that effect of uremic solutes on reactive oxygen species in vitro model system is the method of choice in monitoring the renal function is excessively, will be better written e.g. “…as a possibility in monitoring the renal function”, or the authors better change title to “Effect of uremic solutes on reactive oxygen species in vitro model system”. The renal function is monitored by established clinic-chemical parameters.

Answer:

Following the reviewer suggestion, the manuscript title was changed:

“Effects of uremic solutes on reactive oxygen species in vitro model systems as a possibility of support the renal function management”.

Minor Essential Revisions

Background section

Suggestion 6

P3 L55: change alkoxyl (ROO#) to (RO#)

Answer:

The paragraph containing the cited sentence was removed, since the Reviewer 2 requested a reduction in the “Background” section.

However, we checked all chemical formulas and abbreviations of radical and nonradical species throughout the manuscript.
Results and Discussion section

Suggestion 7

P6 L143: change subtitle to “ABTS#+ radical scavenging by uremic solutes”.

Answer:

The cited subtitle was changed (please see P 12, L 267).

Suggestion 8

P6 L145-147: partly delete and change to “ABTS#+ assay provide a good...”.

Answer:

Considering the changes made in the Discussion section, the cited sentence was removed.

Suggestion 9

P7 L148-149: delete “it was observed that” and beginning with “Uric acid...”.

Answer:

The cited sentence was changed (please see P 12, L 269).

Suggestion 10

P7 L165: ABTS#+ concentration should be given in molar concentration.

Answer:

The cited concentrations, related to Trolox and L-tyrosine, were changed by molar concentration (please see P 15, L 352).

Suggestion 11

P8 L180: change subtitle to “HOCl/OCl- scavenging by uremic solutes”.

Answer:

The cited subtitle was changed (please see P 12, L 276).

Suggestion 12

P8 L192: change subtitle to “O2#- scavenging by uremic solutes”.

Answer:

The cited subtitle was changed (please see P 13, L 286).
Suggestion 13

P9 L207: write Barreiros et al.

Answer:
Done (please see P 17, L 398).

Suggestion 14

P9 L212: change subtitle to “H2O2 scavenging by uremic solutes”.

Answer:
The cited subtitle was changed (please see P 13, L 293).

Suggestion 15

P10 L223: write Chen et al.

Answer:
Considering the changes made in the Discussion section, the cited study was removed.

Suggestion 16

P10 L234-236: write ...showed decreased scavenging capacity against #OH and methyl (#CH3) radicals and singlet oxygen (1O2), increases for O2# and RO# radicals, and no changes for ROO# radical in comparison to healthy individuals.

Answer:
The cited sentence was changed according to the reviewer suggestion (please see P 20, L 467-469).

Suggestion 17

P10 L243: spell out abbreviation LPO.

Answer:
Considering the changes made in the manuscript, the definition of LPO was made in the “Methods” section (please see P 8, L 178).
Suggestion 18

P10 L240: change subtitle to “ROO## scavenging by uremic solutes”.

Answer:

The cited subtitle was changed (please see P 13, L 301).

Suggestion 19

P12 L282: change subtitle to “Oxidant scavenging of uremic solute mixtures”.

Answer:

The cited subtitle was changed (please see P 14, L 320).

Suggestion 20

P13 L300: write Noguer et al. [41].

Answer:

Done (please see P 21, L 485).

Methods section

Suggestion 21

Paragraph P15 L352-356 should be given as first paragraph in section “Results and Discussion”. Paragraph P6 L138-141 should be deleted.

Answer:

The paragraph P 15 L 352-356 was placed as first paragraph in the “Results” section (please see P 12, L 257-262). The paragraph P 6 L 138-141 was deleted.

Suggestion 22

P16 L375: change activity to assay

Answer:

Done (please see P 7, L 146).

Suggestion 23

P16 L386: change activity to assay

Answer:

Done (please see P 8, L 159).
Suggestion 24

P16 L388: citation Ching et al. [57] do not describe the HOCl/OCl- method the authors used, please clarify.

Answer:

The citation Ching et al. [57] was replaced by the following citations:


Suggestion 25

P17 L394: citation [59] do not describe the HOCl/OCl- method the authors used, please clarify.

Answer:

The citation [59] was replaced by the following citation:


Suggestion 26

P17 L402: change subtitle to “Peroxyl radical (ROO#) scavenging assay”.

Answer:

The cited subtitle was changed (please see P 8, L 176).

Suggestion 27

P18 L436: change activity to assay

Answer:

Done (please see P 10, L 227).
**Suggestion 28**

*P18 L483: citation [57] do not describe the H2O2-method the authors used, please clarify.*

**Answer:**

The citation [57] was replaced by the following citations:


**Suggestion 29**

*P19 L451: change subtitle to “Experiments with uremic solute mixtures”.*

**Answer:**

The cited subtitle was changed (please see P 11, L 243).

**Suggestion 29**

Generally, more detailed experimental conditions must be described in methods section. I suggest the authors revise their writing style and try to rephrase parts of the manuscript (e.g.P17).

**Answer:**

Details about the method principles were inserted (please see P 9, L 186-197).

**Figures and figure legends**

**Suggestion 30**

Statistical analysis information are missing, were all test performed in triplicate? Indicated that in figure legends.

**Answer:**

The scavenging capacities against oxidizing species were calculated as the mean of triplicate tests, except for peroxy radical (ROO•) scavenging assay, which was performed in duplicate tests.

This information was placed in the Figure legends.
Suggestion 31

*If all test were performed in triplicate, error bar are not included in the diagrams, in all figures except in Fig. 1B.*

**Answer:**

Error bars were included in all Figures in the new version of the manuscript.

Suggestion 32

*Generally, figures A, B etc. authors described in legends as one figure, but in manuscript all figures are graphically separately. Authors should present figures A, B etc. as one graphic file.*

**Answer:**

The Figures A, B etc. were presented as one graphic file in the new version of the manuscript.

Suggestion 33

*Figure 1. A-C: writing wavelength information for inset in figure legends.*

**Answer:**

The wavelengths were placed in the Figure legends.

Suggestion 34

*Figure 2. A-E: writing wavelength information for inset in figure legends.*

**Answer:**

The wavelengths were placed in the Figure legends.

Suggestion 35

*Description of (v0) and (v) (P30, L728 and L729) should be removed and involved in inset description only, for (v) change to “velocity in the presence of various concentrations of sample”. The Legends for figure 2.D and 2.E are missing.*

**Answer:**

All these changes were made in the Figure legends.
Suggestion 36

*Figure 3.A-B: writing concentration of solute mixtures and wavelength information for inset in figure legends.*

**Answer:**

The concentration of solute mixtures and the wavelengths were placed in the Figure legends.

Suggestion 37

*Figure 4. A-C: writing concentration of solute mixtures and wavelength information for inset in figure legends.*

**Answer:**

The concentration of solute mixtures and the wavelengths were placed in the Figure legends.

Suggestion 38

*Generally, figure legends are rather poor.*

**Answer:**

After the consideration of the reviewer suggestions, figure legends were very improved.
Reviewer 2 (Fumihiro Tomoda)

Comments to the author

Suggestion 1

The authors should read the author guide for preparation of their manuscript and know the instruction for writing the original paper.

Answer:

The manuscript was changed according to the template of “BMC-series medical journals - authors' checklist for manuscript formatting”, which was sent together with the reviewer’s suggestions (http://www.biomedcentral.com/authors/medicine_journals).

Suggestion 2

The section of “Background” was too long to be understood. The background should be succinct.

Answer:

Attempting to diminish and to adequate the “Background”, as well as to make it clear, we have made an extensive change in this section. We believe that these changes improved the quality of the manuscript. The main changes are highlighted in “red”.

Although the “Background” has been shortened when compared with the original manuscript, in this new version it was possible the inclusion of a study by Ujhelyi and collaborators (2006); the authors found that the decreased antioxidant capacity of plasma ultrafiltrate from chronic kidney disease (CKD) patients under hemodialysis (HD) may be due to the dialytic removal of some uremic solutes, increasing the risk of low density lipoprotein (LDL) oxidation and subsequent endothelial cell damage, corroborating with findings about the increase of the oxidative stress in CKD patients after HD.

Cited reference:

Suggestion 3

The author should separate “Results and Discussion” into two sections of “Results” and “Discussion”.

Answer:

“Results and Discussion” was separated into two sections, “Results” and “Discussion”.

Suggestion 4

In Reference 13, age should be included.

Answer:

Considering that “Background” section was changed (please see Suggestion 2), the Reference 13 was removed in the new version of the manuscript.
Reviewer 3 (Hidehisa Shimizu)

Major Compulsory Revisions

Question 1

The authors chose uremic solutes to check antioxidant effects in the study. Why were these uremic solutes chosen? The authors should be described.

Answer:

A broad set of rationale was used to choice the uremic solutes investigated in this manuscript. The need to include uremic solutes classified according to their size and binding properties was one criterion. Vanholder and collaborators (2003), on behalf of the “European Uremic Toxin Work Group”, they published a systematic overview of the most studied uremic solutes between 1966 and 2002 in more than 850 publications; most studies were performed in patients undergoing chronic hemodialysis (HD) and in non-dialyzed chronic kidney disease (CKD) patients. So, according to the size and binding properties, the authors observed that the most studied uremic solutes were classified into three major classes:

- free water soluble low molecular mass compounds (<0.5 kD);
- middle molecules (0.5-60 kD);
- protein-bound solutes.

In free water soluble low molecular solutes, it can be found the uremic solutes uric acid (purine group), methylguanidine (guanidine group), creatinine (guanidine group) and urea, these two last also choice as commonest uremic solutes.

Protein-bound solutes included hippuric acid (hippurate group), phenol and p-cresol (phenol group) and indoxyl sulfate (indole group).

Middle molecules were not chosen since they included proteins, which were not of interest in our study.

The great number of uremic solutes in each class makes us to establish other criteria to choice the compounds. In this way, the second criteria was the highest \( \frac{C_U}{C_N} \) ratio of uremic solute, also presented by Vanholder et al. (2003), where \( C_U \) is the mean/median uremic concentration, and \( C_N \) is the normal concentration of the uremic solute. Considering this criterion, the uremic solutes that showed values of \( \frac{C_U}{C_N} > 15 \) were methylguanidine...
(free water soluble low molecular solute, guanidine group), **indoxyl sulfate** (protein-bound solute, indole group), **hippuric acid** (protein-bound solute, hippurate group) and **p-cresol** (protein-bound solute, phenol group).

Although **uric acid** showed a low $C_u/C_n$ value (1.24), this solute is a well-known, powerful biological antioxidant, which increased circulating levels in humans has been cited as a protective mechanism against lipoperoxidation in conditions of cardiovascular diseases (Nieto et al., 2000).

**Creatinine** (free water soluble low molecular solute, guanidine group) and **urea** (free water soluble low molecular solute) were chosen in our study since they are commonest uremic solutes.

In addition, several evidences about the antioxidant potential of the uremic solutes have been found. Ujhelyi and collaborators (2006) demonstrated that plasma ultrafiltrate of CKD individuals had antioxidant activity, inhibiting LDL oxidation (*in vitro* assay), and that this antioxidant capacity was lost after HD, as a consequence of the dialytic removal of some compounds, such as **indoxyl sulfate**, **p-cresol**, **phenol**, and **uric acid**; the retention of other solutes, including **L-arginine**, **creatine**, **guanidine**, and **hippuric acid**, among others, was not sufficient to prevent the oxidative modification of LDL. Miyamoto et al. (2010) also observed that **indoxyl sulfate**, **uric acid** and **p-cresol** had antioxidant activity within the concentration range for non-CKD conditions, exhibiting a potent ability to scavenge superoxide anion ($O_2^{•–}$) comparable to that of superoxide dismutase. These findings stimulate us to choose these solutes to investigate their antioxidant in vitro potential (please see also the Answer of Question 3).

Considering this reviewer question, we have included a sentence in the “Results” section to explain why we have chosen these uremic solutes (please see page 12, lines 262-265).

**Cited references:**


**Question 2**

*In the patients of CKD and HD, one of the highest uremic solute in serum is indoxyl sulfate. Why did the authors not check antioxidant effects of indoxyl sulfate?*

**Answer:**

Indoxyl sulfate is an important protein-bound uremic solute, which increased circulating levels have serious deleterious effects in various cell types, including renal tubular cells (Motojima et al., 2003), vascular endothelial cells (Dou et al., 2004), vascular smooth muscle cells (Muteliefu et al., 2012), among others. Also, it is well known the association of indoxyl sulfate with impairment of renal function and development of cardiovascular disease in CKD patients (Barreto et al., 2009; Wu et al., 2011; Lin et al., 2012).

In addition, the antioxidant potential of biological samples has been attributed to the presence of indoxyl sulfate (Ujhelyi et al., 2006). Using *in vitro* systems for the generation of superoxide anion, \( \text{O}_2^\cdot \) (xanthine/xanthine oxidase system and activated neutrophils) and hydroxyl radical, \( \cdot \text{OH} \) (H\(_2\)O\(_2\)/UV system), Miyamoto and colleagues (2010) found a great potential of indoxyl sulfate in capturing these reactive species, corroborating the findings on the antioxidant activity of biological samples rich in this uremic solute.

For all these reasons, indoxyl sulfate has always been among the uremic solutes chosen for our study. However, until this moment, we did not have available any of the indole derivatives. In fact, beyond the indoxyl sulfate, we think should be included other indole compounds, such as 3-indoleacetic acid, 5-hydroxy-3-indoleacetic acid, indoxyl-beta-D-glucuronide.
Despite this problem, we believe that we have demonstrated the use of analytical parameters such as the IC\textsubscript{50} through \textit{in vitro} model systems, since there is now available a specific monitoring antioxidant effects on ROS of interest in CKD (see model proposed by Oowada et al., 2012).

**Cited references:**


**Question 3**

*There are reporting that L-arginine is a useful molecule to prevent life-style diseases. Therefore, it is possible that L-arginine also prevents progression of CKD and HD through antioxidant effects. However, p-cresol is a typical uremic toxin and induces production of reactive oxygen species (ROS) in some tissues. The authors should discuss the character of p-cresol, along with the present results, in the patients with CKD and HD.*

**Answer:**

Accumulation of p-cresol in CKD has been often associated with the onset of uremia-related cardiovascular diseases and kidney complications by mechanisms involving oxidative stress (Watanabe et al., 2013; Chang et al., 2014). However, from a structural perspective, p-cresol and also other uremic solutes, such as uric acid, indoxyl sulfate, may exhibit antioxidant capacity.

Miyamoto et al. (2010) showed that p-cresol have a potent antioxidant activity, comparable to that of superoxide dismutase, assessed by luminal chemiluminescence, which represents the ability to scavenge $O_2^\cdot$. In a study by Ujhelyi and collaborators (2006a), it was demonstrated that plasma ultrafiltrate (molecular weight < 5000 Da) of CKD individuals exhibited i) pronounced antioxidant activity, assessed by the ability to inhibit the heme-mediated LDL oxidation (*in vitro* assay), and ii) protection against endothelial cytotoxicity induced by LDL oxidation. This antioxidant capacity of plasma ultrafiltrate from CKD patients was lost after HD, as a consequence of the dialytic removal of some compounds, including the uremic solutes indoxyl sulfate, p-cresol, phenol, and uric acid; in addition, it was observed that the retention of other solutes, including L-arginine, creatinine, guanidines, hippuric acid, among others, was not sufficient to prevent the oxidative modification of LDL.

Interesting to note that many of the uremic solutes investigated about their antioxidant potentials in our study corroborated the findings of Ujhelyi et al. (2006a). According our data, p-cresol, phenol and uric acid were effective antioxidants in three model systems (ABTS$^{**}$, HOCI/OCl$^\cdot$, crocin bleaching assay); according Ujhelyi et al. (2006a), these solutes may be participating in the antioxidant activity of plasma against *in
vitro LDL oxidation. Some compounds cited by Ujhelyi and colleagues (2006a) were ineffective in preventing LDL oxidation, such as creatinine, hippuric acid, methylguanidine and L-arginine, and in our study they did not have antioxidant activity or they showed a minor efficiency as antioxidants.

In front of this, and citing a sentence of Ujhelyi and colleagues in response to Meijers (Ujhelyi et al., 2006b), “p-cresol can be considered to be a Janus-faced compound with several toxic and some beneficial properties, and it might be a marker of pathologic metabolic processes that lead to the observed enhanced risk of mortality in hemodialysis patients”.

Although the Discussion section has been changed, as suggested by the Reviewer 2, in this new version of the manuscript the studies by Ujhelyi and collaborators (2006a) and Miyamoto and collaborators (2010) were included, to comply with the suggestion about the discussion of our data with p-cresol, in the patients with CKD and HD, but we took the opportunity to extend the discussion to other uremic solutes. Please see Discussion section, page 18, lines 407-413; page 19, lines 435-451.

We also included L-arginine in our study based on extensive literature reporting the beneficial effects of L-arginine supplementation in various experimental models of CKD, including diabetic nephropathy, hypertensive nephrosclerosis, and partial nephrectomy, among others (Reyes et al., 1992; Chen et al., 1993; Reyes et al., 1993; Cherla and Jaimes, 2004).

The main mechanism by which L-arginine leads to beneficial effects on CKD has been attributed to the increased NO production, since nitric oxide (NO) synthase (NOS) activity in kidney failure is determined by L-arginine concentration (Schmidt et al., 1999); so, L-arginine in CKD is important to oppose endothelial dysfunction. In a review by Baylis (2006), it is pointed that the total NO production is decreased in CKD patients due to main possible causes: i) the limitation of substrate (L-arginine) for nitric oxide (NO) synthase (NOS), and ii) the increased levels of circulating endogenous inhibitors of NOS, particularly asymmetric dimethylarginine (ADMA). Evidence indicates that low levels of NO contribute to cardiovascular events and progression of kidney damage in CKD individuals, such as hypertension and focal glomerulosclerosis, the hallmark of progressive CKD (Klahr, 2001; Modlinger et al., 2004; Baylis, 2012). In addition, increased circulating levels of ADMA have been found in CKD (Aldámiz-Echevarría and Andrade, 2012), as a
consequence of both decreased rate of ADMA catabolism by dimethylarginine dimethylamino hydrolase (DDAH) and increased rate of ADMA synthesis by type I protein arginine methyltransferase (PRMT). DDAH activity is reduced by oxidative stress in CKD and end-stage renal disease (Tain and Baylis, 2007), while type I PRMT activity is increased in endothelial cells under conditions of oxidative stress and increased oxidized LDL levels (Böger et al., 2000).

Despite the positive outcomes of the L-arginine supplementation in experimental models of CKD, these evidences have not been completely translated into the human conditions of renal disease; studies have shown that neither acute nor chronic L-arginine administration improved arterial endothelial function in adults or children with CKD (Cross et al., 2001; Bennett-Richards et al., 2002), although acute L-arginine supplementation improves the impairments in the venal dilatation responsiveness (induced by acetylcholine) in end-stage renal failure subjects (Hand et al., 1998). In front of this, more studies are necessary to comprehend the consequences of L-arginine supplementation, as well as the mechanisms by which this amino acid could bring benefits against human renal diseases.

Considering the possibility that the beneficial effects of L-arginine on CKD could be reached by counteracting oxidative stress, since the increase in ADMA levels is a consequence of changes in the activities of DDAH and type I PRMT promoted by oxidative damage, it becoming interesting the study of the antioxidant potential of L-arginine in CKD states. However, according our present data, L-arginine seems not to be an effective antioxidant, because the concentration of this amino acid needed to scavenge 50% of ABTS** and HOCl/OCI was the highest among all the studied compounds, and also L-arginine did not showed activity against O2•-, H2O2 and ROO• (crocin bleaching assay), at any tested concentration.

Our findings are in agreement with Adams et al. (1999), which assessed the antioxidant potential of L-arginine using various assays, including i) anti-tocopherol mediated peroxidation (in vitro assay), which tests the ability of the antioxidant to synergize with alpha-tocopherol to prevent chemically-induced LDL oxidation, and ii) lipid peroxide levels after L-arginine administration (ex vivo assay). L-arginine did not show significant antioxidant effects, using either in vitro or ex vivo approaches; no changes were observed in lipid peroxide levels following acute L-arginine administration. The authors suggested that L-arginine has not considerable antioxidant properties, and that its beneficial cardioprotective effects may occur through mechanisms depending on NOS pathway.
Similarly, Mendoza et al. (2008) observed that the treatment of nephrectomized (two-thirds renal ablation of the left kidney) mice with a combination of L-arginine and antioxidants (vitamins C and E) omega-3 fatty acids ameliorated various parameters of kidney failure, reducing proteinuria, blood pressure and superoxide anion production; the amelioration of renal disease was attributed to a combination between the superoxide scavenger capacity of the antioxidants and the enhanced NO availability due to arginine supplementation, since the authors suggested that NO represents a defense against oxidative stress during the development of kidney failure.

Although the Discussion section has been changed, as suggested by the Reviewer 2, in this new version of the manuscript the studies by Schmidt et al. (1999) and Baylis (2006) were included, to bring information about the beneficial role of L-arginine supplementation to CKD patients. Please see Discussion section, page 17, lines 389-396.

Cited references:


