Author's response to reviews

Title: Time course study of oxidative and nitrosative stress and antioxidant enzymes in K2Cr2O7-induced nephrotoxicity

Authors:

Jose Pedraza-Chaverri (pedraza@servidor.unam.mx)
Diana Barrea (dianabarrera@hotmail.com)
Omar N Medina-Campos (mconoel@servidor.unam.mx)
Raymundo C Carbajal (clementecarbajal@hotmail.com)
Rogelio Hernandez-Pando (rhdezpando@hotmail.com)
Norma A Macias-Ruvalcaba (normaamr@hotmail.com)
Perla D Maldonado (m_cdeyanira@yahoo.com)
Marcos I Salcedo (ni_be_pb@hotmail.com)
Edilia Tapia (edilitapia@hotmail.com)
Liliana Saldivar (saldivar@servidor.unam.mx)
Maria E Castilla (elenacm@servidor.unam.mx)
Maria E Ibarra-Rubio (meir@servidor.unam.mx)

Version: 3 Date: 19 February 2005

Author's response to reviews: see over
February 18, 2005

The Editorial Team
Bio Med Central

Re. MS: 2049866521502504 - Time course study of oxidative and nitrosative stress and antioxidant enzymes in K2Cr2O7-induced nephrotoxicity

Dear members of the Editorial Team:

Enclosed please find the revised version of our manuscript ‘Time course study of oxidative and nitrosative stress and antioxidant enzymes in K2Cr2O7-induced nephrotoxicity’. We hope this revised version be suitable for publication in BMC Nephrology

Sincerely

José Pedraza-Chaverri
Facultad de Química, Edificio B
Lab 209, 2o. Piso
Ciudad Universitaria, UNAM
04510 México, D.F.
México

Phone and fax 52-55-5622-3515
Email: pedraza@servidor.unam.mx

Our point-by-point-response is as follows:

POINT-BY-POINT RESPONSE

Reviewer Kanwaljit Chopra: 1 February 2005

Q1. Authors must indicate the method of collection of blood. They should also report the mortality data in each group.
R. The rats were decapitated and the blood was obtained from trunk. This is indicated in the text (page 6). There was mortality.

Q2. What is the justification that there is no alteration of SOD activity whereas all the other enzymes decrease significantly after K2Cr2O7 administration?
R. This is a very interesting point. We do not have the exact answer. However, we observed immunostaining of both Mn-SOD and Cu,Zn-SOD proteins in cell detritus suggesting that this may explain the absence of changes in SOD activity in K2Cr2O7-treated animals.

The following sentence was added to page 14:
“This may be consequence of the fact that both SOD enzymes were immunolocalized even in the damaged epithelial tubular cells.”

Q3. Few of the spelling mistakes have to be corrected (e.g. page 12, 2 Para, 5 line immunostaing??)
R. immunostaing was changed to immunostaining
Q1. The conclusions are long and should be concise and may be modified as: The data show that the association between oxidative/nitrosative stress and not the antioxidant enzymes with functional and structural renal damage induced by K2Cr2O7 in rats at different time points.
R. The following phrase was added as a conclusion (page 14).
“The data show an association between oxidative/nitrosative stress and functional and structural renal damage induced by \( \text{K}_2\text{Cr}_2\text{O}_7 \) in rats at different time points, but not between this damage and antioxidant enzymes.”

Q2. Background: The authors are suggested to move the first three sentences from “Results and Discussion” section on page 10 of the manuscript to the beginning of the Background on page 4.
R. This change was performed in the text. The references were arranged accordingly.

Q3. The last three sentences in Background on page 5 should be removed.
R. These sentences were removed.

Q4. There are earlier reports pertaining to the dose and time response of K2Cr2O7–induced nephrotoxicity (biochemical and histological changes) in rats (Gumbleton and Nicholls, Food Chem. Toxicol. 26 (1): 37-44, 1988; Nagaha E.O. Gen. Pharmacol. 12(6): 497-500, 1981). Authors are suggested to include these reports in Background and Discussion of the manuscript.
R. These two references were included in Background and Discussion. The references were arranged accordingly. The number of these references are 10 and 11 (page 17).

Q5. Results and Discussion: This portion of the manuscript reveals more results and less discussion reviewer suggests more discussion in light of the present data.
R. The discussion was expanded in several specific points as requested by the referees. The paragraphs/sentences added to this section are inserted in response to specific questions in this point-by-point response letter.

Q6. First three sentences should be moved to Background.
R. These three sentences were moved to the background (page 4).

Q7. Page 12: last para, first sentence, to be modified as “Further we investigated the time response of the renal antioxidant enzymes in K2Cr2O7-treated rats.”
R. The sentence was modified as suggested (page 14).

Q8. Page 13: Para 1, last sentence, Explain why these enzymes remained low in spite of abolished oxidative/nitrosative stress.
R. The following paragraph was added at the end of the discussion (page 13) to address this point.
“The reason why these enzymes remained low at the end the study is not clear, however we are tempting to speculate that proximal tubules have not reached still the full capacity to synthesize these enzymes and/or
factors other than oxidative/nitrosative stress are involved in the diminution in these enzymes. Therefore, additional studies are required to explain why some antioxidant enzymes remained low on days 10-12 in absence of oxidative/nitrosative stress.”

---

**Minor Essential Revisions**

**Q9. Abstract:** Authors stated that “serum and kidney chromium content increased reaching the highest value on day 1” but what was the value from 0-20 hrs after K2Cr2O7 administration?

R. Unfortunately, chromium content was not measured at 0-20 hs.

**Q10.** “Activity of GR decreased on days 2-10” but in the background it is stated that “renal activity GR remained unchanged at 24 and 48 h”

R. The data reported in the background were obtained in a previous work (Life Sci. 2003;73(23):3027-41) in rats with higher body weight than those used in the present work (290-310 g vs. 200-210 g in the present work). In this context it has been shown that the nephrotoxic effect of K2Cr2O7 is age-dependent [Appenroth D, Braunlich H. Age dependent differences in sodium dichromate nephrotoxicity in rats. Exp Pathol. 1988;33(3):179-85]. In addition, the volume of administration of K2Cr2O7 solution was different (1.0 ml in the former publication vs. 0.5 ml in the present work). This may explain the differences in GR activity in both protocols.

**Q11. Methods:** Why the authors did not use monoclonal instead of polyclonal antibodies for specificity?

R. Polyclonal antibodies used in this work were specific. Positive and negative controls with these polyclonal antibodies were satisfactory.

**Q12. Did the authors find any cross reactivity using polyclonal antibodies?**

R. No we did not find any cross reactivity with the polyclonal antibodies. We observed a single band in the western blot analysis for catalase, Mn-SOD and Cu,Zn-SOD.

**Q13. The age of the animals and total number of rats used in each group is missing and exactly how many rats from each group were sacrificed at 8 different time points?**

R. In the present work we used seven-week-old rats. The number of rats used in each time point is indicated in the following table.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>K2Cr2O7</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

In some cases, the number of determinations was lower than the number of rats sacrificed due to the fact that the sometime the samples were not sufficient to perform all determinations.

There was no mortality.

The respective paragraph page 6 (experimental design) was modified to include the above data as follows:

“Seven-week-old male Wistar rats with an initial body weight of 200-210 g were used. Experimental work was approved by CONACYT (#25441) and DGAPA (IN210201) and followed the guidelines of Norma Oficial Mexicana (NOM-ECOL-087-1995). Two groups of rats were studied: 1) CT, control injected subcutaneously with 0.5 ml isotonic saline solution (n=40); and 2) K2Cr2O7, treated with a single subcutaneous injection of 15 mg/Kg K2Cr2O7 [7] in a volume of 0.5 ml (n=43). The study was performed in two stages: rats from days 1,2,3,4, and 6 were studied in the first one (n=5/per group) and rats from days 8, 10, and 12 were studied in
the second one (n=5 for control group and n=6 for K$_2$Cr$_2$O$_7$ group). Rats were sacrificed on days 1, 2, 3, 4, 6, 8, 10, and 12. There was no mortality

In addition, The urinary determinations on days 1-10 were higher than the number of rats sacrificed due to the fact that urine samples from rats sacrificed later were included. For example, urine of rats sacrificed on days 8, 10 and 12 were collected also on days 1, 2, 3, 4, and 6 and used for the urinary determinations of these days.

In the following table we can see the number of urinary determinations in each time point,

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>K$_2$Cr$_2$O$_7$</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>16</td>
<td>16</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

Q14. From which tissues/organs the blood was collected i.e. from heart, orbital plexus or jugular vein?
R. The rats were decapitated and the blood was obtained from trunk. This is indicated in the manuscript (page 6).

Q15. In “western blot” w should be capitalized.
R. “western blot” was changed to “Western blot”

Q16. How many animals in each group used for metal analysis, histology, immunohistochemistry, Western blot and antioxidant enzyme assays?
R. The number of animals used is indicated above. We used a single animal to perform all determinations. In some exceptional cases, the sample was not sufficient to perform all determinations (please see the response to Q13).

Q17. For chromium analysis whole kidney or corticomedullary region was used.
R. Whole kidney was used to perform chromium determination. To clarify this in the text (chromium concentration section) we added the word “total” before kidney (page 7).

Q18. The spelling of “hematoxilin” needs to be checked.
R. “hematoxilin” was changed to “hematoxylin” (pages 7 & 8).

Q19. For antioxidant enzyme activity assay how the tissue homogenate was prepared?
R. The following phrase, to describe the method to prepare renal homogenates, was added starting the paragraph “Activity of antioxidant enzymes” (page 9):
“Kidney was homogenized in a Polytron (Model PT 2000, Brinkmann, Westbury, NY, USA) for 10 seconds in cold 50 mM potassium phosphate, 0.1% Triton X-100, pH = 7.0. The homogenate was centrifuged at 19,000 x g and 4°C for 30 min and the supernatant was separated to measure total protein and the activities of CAT, GPx, GR, and total SOD”

Q20. What was the rationale for only CAT protein analysis by Western blot?
R. We also performed Western blot analysis for Mn-SOD and Cu,Zn-SOD. The following paragraphs were added to describe this addition in methods (page 10) and in results/discussion (page 14) sections:
Immunodetection was performed using specific primary antibodies against CAT (1:500 dilution), Mn-SOD (1,500 dilution), or Cu,Zn-SOD (1:5,000 dilution). Membranes was then probed with the appropriate secondary antibody-peroxidase conjugate (1:5,000 dilution).

These data are in agreement with kidney total SOD activity (Fig. 9) and with the protein content of Mn-SOD and Cu,Zn-SOD measured by Western blot which remained unchanged at all time points in K$_2$Cr$_2$O$_7$-treated rats (data not shown).”

We did not perform western blot for glutathione peroxidase and glutathione reductase because reliable antibodies were unavailable in our laboratory.

Q21. The spelling of “polyacrilamide” needs to be checked on page 9.
R. “polyacrilamide” was changed to “polyacrylamide” (page 10).

Q22. Page 10: Para 1, last sentence “In both cases-------on day 6.” The pharmacokinetics/pharmacodynamics of chromium needs to be discussed in light of the published reports.
R. The following sentence was added at the end of the first paragraph of page 11: “Our data are consistent with previous pharmacokinetic studies which have shown that chromium is rapidly distributed [45] and that the half life of chromium is longer in kidney than in blood serum [46].”

Q23. Page 11: Para 1, last sentence, explanation is needed to improve the statement.
R. The following paragraph was added to the pages 11 & 12:
“This may be explained by the fact that chromium (VI), which is readily taken up into tissues, is reduced inside the cell to the final stable product chromium (III) [47]. The biological effects of chromium (VI) are generally attributed to cellular uptake, because chromium (VI), in contrast to chromium (III), is easily taken up by cells through the sulfate anion transport system [48,49]. However, once inside, chromium (VI) is reduced through reactive intermediates such as chromium (V) and chromium (IV) to the more stable chromium (III) by cellular reductants including glutathione, vitamins C and B$_2$, and flavoenzymes [48]. Thus, the formation of chromium (III) or other intermediate oxidation states, in particular chromium (V), is believed to play a role in the biological effect of chromium (VI) compounds. In vitro studies have shown that this reduction process causes the generation of active oxygen species [50] which are involved in renal damage [16,35]. Interestingly, it has been shown that a low dose of K$_2$Cr$_2$O$_7$ (10 mg/Kg) is unable to induce nephrotoxicity suggesting a threshold of this compound to induce renal damage [10]. In addition it is known that chromium is located in vacuoles inside the proximal tubular cells which may delay the excretion of this metal [51]. In fact it has been shown that chromium remains for a long time in several tissues including kidney [45,46,51] which is consistent with our data. “

POINT-BY-POINT RESPONSE

Reviewer’s report
Date: 7 January 2005 Version: 2
Reviewer: Leonard Rybak

General
Q1. Was the dose of K2Cr2O7 selected on the basis of previous studies indicating that this is an appropriate nephrotoxic dose of chromium to employ?
This dose was selected on previous studies (Clin Invest Med 1995, 18:424-434, Free Radic Biol Med 2003, 34:1390-1398, Food Chem Toxicol 1988, 26:37-44). In addition, it has been shown that K$_2$Cr$_2$O$_7$ at 10 mg/Kg is unable to induce nephrotoxicity (Food Chem Toxicol 1988, 26:37-44).

Q2. What was the total number of rats used in the study, and how were the numbers per group decided upon?
R. The total number of rats was: 40 control and 43 K$_2$Cr$_2$O$_7$-treated rats. The number of rats in each group (n=5/day in control and n=5-6/group in K$_2$Cr$_2$O$_7$ group) was chosen taken into account that the experimental model is reproducible and this was a time course study (8 time points). This is clarified on page 6.

Q3. What is the sensitivity of the atomic absorption spectrophotometric method for the detection of chromium?
R. 5 µg/L

Q4. Presumably, it does not distinguish among various metabolites and reduction intermediates of the parent compound. This makes it difficult to make firm conclusions about the association between chromium concentrations in tissues and the toxic effects measured. For example, the authors state on p. 11, â€œInterestingly, the renal damage disappeared in spite of the kidney still had high levels of chromium on days 8-12.â€ They do not discuss why this is the case. They should consider that chromium at these time points is present in the kidney tissues in some bound form that is nontoxic, that it has been converted into a nontoxic metabolite, that chromium itself induced the formation of heme-oxidase-1, that the level, although significantly elevated above control is below the threshold for nephrotoxicity, or other possible mechanisms to explain this finding.
R. Yes, the method used to measure chromium content is unable to distinguish among the different chromium metabolites. The following paragraph was added to page 11 & 12 to expand our observations: “This may be explained by the fact that chromium (VI), which is readily taken up into tissues, is reduced inside the cell to the final stable product chromium (III) [47]. The biological effects of chromium (VI) are generally attributed to cellular uptake, because chromium (VI), in contrast to chromium (III), is easily taken up by cells through the sulfate anion transport system [48,49]. However, once inside, chromium (VI) is reduced through reactive intermediates such as chromium (V) and chromium (IV) to the more stable chromium (III) by cellular reductants including glutathione, vitamins C and B$_2$, and flavoenzymes [48]. Thus, the formation of chromium (III) or other intermediate oxidation states, in particular chromium (V), is believed to play a role in the biological effect of chromium (VI) compounds. In vitro studies have shown that this reduction process causes the generation of active oxygen species [50] which are involved in renal damage [16,35]. Interestingly, it has been shown that a low dose of K$_2$Cr$_2$O$_7$ (10 mg/Kg) is unable to induce nephrotoxicity suggesting a threshold of this compound to induce renal damage [10]. In addition it is know that chromium is located in vacuoles inside the proximal tubular cells which may delay the excretion of this metal [51]. In fact it has been shown that chromium remains for a long time in several tissues including kidney [45,46,51] which is consistent with our data."

Q5. The method to quantitate the percentage of tubules with histopathological alterations is unclear. Did the investigators measure damaged tubules per high power field to generate a percentage of tubular damage, or how did they obtain a percent value?
R. The method is described in the text. For example, for each group and at each time point 100 proximal tubules surrounding glomeruli of external renal cortex were counted (20 tubules/rat, n=5). The number of tubules with histopathological alterations were identified and then the percentage of damaged tubules was calculated.

To clarify this point the paragraph to describe this method was modified in the text (page 7) as follows: “A quantitative histological damage was determined by using a Leica Qwin Image Analyzer (Cambridge, UK). The histological profile of twenty proximal tubules randomly selected per rat (5 rats per group) was recorded (n=100 tubuli/group at each time point). The number of tubules with histopathological alterations like swelling,
cytoplasmic vacuolization, desquamation or necrosis was registered and the data were expressed as percentage of damaged tubules. The percentage of damaged tubules of K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} and control groups was compared.

---

Q6. Were the evaluators of the tubular damage blinded to the treatment?
R. No the evaluator was not blinded to the treatment.

Q7. The data in Figure 4 are not presented in a consistent manner. Some of the micrographs are reported in the figure legend as being 400X (A-C). However, it is obvious that other micrographs shown in this figure vary in magnification. Is there some justification for this variation? The actual magnification for each panel should be specified.
R. The magnification in all cases in Figure 4 was 400 X. This is stated now in the corresponding figure legend (page 22).

Q8. In 4H, the authors state that there was “very strong” immunostaining for DNP. However, the micrograph presented appears to show moderate staining at best, and the cells appear to be just membrane ghosts in H and I.
R. To clarify more precisely this point we performed a quantitative image analysis to estimate the stained area. The data are presented in Table 1 (page 24). We can see that DNP and 3-NT immunostaining was significantly different from day 0. In the case of DNP the increase on day 2 was 1.7 fold compared with day 0. The phrase “very strong” was changed to the word “clear” in the text (page 12) and in the figure legend (page 22).

Furthermore, the following sentence was added at the end of the section: “Immunohistochemical localization of 3-NT, protein carbonyls, Cu,Zn-SOD, Mn-SOD, and CAT” (page 8) to describe briefly the method to quantitate the stained area:
“The stained area was quantified using a SigmaScan Pro (version 4.01.003) (Jandel Scientific, San Rafael, CA). The data are expressed as percent respect to day 0.”

Finally, it is possible that the different appearance of the slides (like ghost membranes) may be secondary to the fact that these slides were submitted to a previous treatment before the immunostaining (incubation with 0.2% DNPH in 2 N HCl for 60 min at room temperature in absence of light). This had been previously described in methods (page 8).

Q9. The micrographs in Figure 7 also vary in magnification.
R. The magnification for the panels A-C and G-I is 100X, and for the panels D-F is 400X. This is now stated in the figure legend (page 23).

Q10. The authors in the figure legend for 7C appropriately state that strong CAT immunoreactivity was observed in the renal tubular epithelium 12 days after K2Cr2O7 administration. Actually, it appears that the immunostaining is more intense in these tubules than that observed in controls. Did the authors notice some sort of “erebound” phenomenon whereby the CAT immunoreactivity in the kidneys of rats recovering from chromium-induced acute tubular necrosis was greater than it was in untreated controls?
R. We performed a quantitative analysis of the stained area of several rats (please see table 1). In fact, it can be seen that the stained area of CAT on day 12 is higher than on day 0, however this value is not significantly higher than that observed on day 0.

Q11. In the immunostained micrographs for Cu/Zn SOD and Mn-SOD, it appears that there are differences in the intensity of immunostaining that is not described by the authors. It looks like the intensity of
immunostaining for Cu/Zn-SOD was greatest 2 days following chromium treatment (E), of intermediate intensity 12 days after treatment (F), and of lowest intensity in controls (D).
The intensity for immunostaining for Mn-SOD seems greatest in controls (G), of intermediate intensity in kidney from rats 2 days after treatment (H) and lowest in rat kidneys 12 days after treatment (I).
R. The quantitative analysis of the stained area showed no significative changes in both Cu,Zn-SOD and MnSOD (please see table 1 on page 24).

Q12. The last sentence of the body of the manuscript on p.13 under conclusions does not make sense, however. This sentence needs to be restated for clarity. I think that the authors are trying to state that the decrease in the urinary excretion of NO metabolites Was related to protein nitration that would result in a reduced elimination of nitrite and nitrate in the urine.
R. The sentence was rewritten as suggested by another reviewer as follows (page 14):
“The data show an association between oxidative/nitrosative stress and functional and structural renal damage induced by \( K_2Cr_2O_7 \) in rats at different time points, but not between this damage and antioxidant enzymes.”

Q13. There are numerous other sentences that lack clarity and need to be edited. General examples are the statements like “along the study” (p.13 and in other parts of the manuscript),
R. “along the study” was changed to “at all time points” (pages 8, 10, 13 and 14)

Q14. Regarding to the SOD enzymes (p. 12)
R. The phrase “Regarding to the SOD enzymes” was eliminated.

Q15. and in the figure legends and text stating “after 12 days of treatment.” The latter implies that the rats were treated for 12 days with chromium, whereas they only received a single dose on day 1.
The following phrase was added to the figure legends 4 and 7 (page 22) to clarify when the studies were performed: “The study was performed in control rats (day 0) and on days 2 and 12 after a single injection of \( K_2Cr_2O_7 \) (15 mg/Kg).”
Taking into account that this phrase was inserted at the beginning of the figure legend and to avoid confusion, the phrases “after 12 days of \( K_2Cr_2O_7 \) administration” or “after 2 days of \( K_2Cr_2O_7 \) administration” were changed to “on day 12” or “on day 2”, respectively (page 22).

Q16. The two lines preceding the Conclusions section (p.13) do not form a complete sentence.
R. The two lines were rewritten as follows (page 14):
“Interestingly GPx, GR, and CAT remained low on day 10 an GPx remained low on day 12 in absence of oxidative and nitrosative stress.”

Finally, the following references were added to the manuscript (page 20). We had to do that to support some paragraphs added to the discussion.


