Author's response to reviews

Title: Effects of iodinated contrast agents on renal oxygenation level by BOLD-MRI in a rabbit model of diabetic nephropathy

Authors:

Jia-huan Wang (wangjiahuan.2008@163.com)
Ke Ren (zhongguoyidarenke@163.com)
Wen-ge Sun (wengesun_zgyd1@163.com)
Li Zhao (m15840597875@163.com)
Hong-shan Zhong (wangjiahuan_2008@yeah.net)
Ke Xu (zhongguoyidaxuke@126.com)

Version: 4 Date: 6 July 2014

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

Title: Effects of iodinated contrast agents on renal oxygenation level by BOLD-MRI in a rabbit model of diabetic nephropathy

Authors:
Jia-huan Wang, PhD, MD, Email: wangjiahuan.2008@163.com
Ke Ren, PhD, MD, Email: zhongguoyidarenke@163.com
Wen-ge Sun, MD, Email: wengesun_zgyd1@163.com
Li Zhao, MD, Email: m15840597875@163.com
Hong-shan Zhong, PhD, MD, Email: wangjiahuan_2008@yeah.net
Ke Xu, PhD, MD, Email: zhongguoyidaxuke@126.com

Version: 1 Date: July 5, 2014
Author's response to reviews: see over
Reviewer's report
Title: Effects of iodinated contrast agents on renal oxygen level by BOLD MRI in a model related to rabbits' diabetic nephropathy
Version: 6 Date: 7 March 2014
Reviewer: Robert Menzies

Reviewer's report:
This manuscript investigates the question of whether iodine based contrast agents can alter the BOLD signal. This is an important research question with potentially important clinical implications as has been previously explored by others, for example Heneder et al. (Invest Radiol. 2012 May;47(5):299-305). The authors present a large and commendable dataset with strong group numbers. However a number of major revisions must be made to the manuscript, and potentially additional experiments must be performed, to raise it to a publication standard as outlined below.

• Major Compulsory Revisions
There are many grammatical errors in this manuscript. These cannot be accurately referred to for correction until the authors provide a manuscript with page/line numbers. The manuscript would also benefit from critical reading to address multiple basic grammar errors.

We have changed the grammatical errors in this manuscript and provided the manuscript with page/line numbers.

Abstract:
The BOLD signal relates to oxygenation status but is not an absolute measure of tissue pO2. To be clear “renal oxygen levels” should really be changed to “renal oxygenation levels”. This should also be made clear throughout the manuscript.

Change made as indicated by the reviewer.

Background:
What qualifies hypoxia as a hot topic in in DN? The reference given does not support the argument since Rosenberger et al. propose that STZ treated rats show significant resistance to hypoxia induced injury. The study of Nordquist et al. (Adv Exp Med Biol. 2013;765:185-93), for example, provides one such recent and investigation of the consequences of renal hypoxia following diabetes that should be considered by the authors. Such mechanistic insights may equally serve well to strengthen the discussion.

Thank you for your advice. The reference surely does not support the argument. In order to avoid misunderstanding, we have rewritten the background of the manuscript and delete the sentence and then added new references.

Methods:
Why were no untreated (i.e. non diabetic) rabbits given the 350 contrast agent? Or indeed why were such control animals not used for the dosing experiment to identify a peak dose to then be applied to the experimental treatment (type II and I diabetic) groups?

We have added the control groups in the experiment.

Similarly, have the authors investigated what occurs under higher contrast agent doses (e.g. 400 + mg I/ml)? Investigating a higher dose range (for both type I and II diabetic animals) might also help to find differences between the treatment groups in terms of R2*.

Thank you for your advice. In this experiment, we have used four different iodinated contrast agents. Both of them were provided by GE company and have a good consistency. Meanwhile, the
four different iodinated contrast agents were used very common in clinical. About the higher contrast agent doses (e.g. 400 + mg I/ml), we have never used in CT contrast enhancement in clinical. Please give me a chance to review my manuscript again, if the manuscript does not have other questions, we would to provide relevant information immediately.

Were all TE’s used to construct the T2* map? If so, then by what factor is the final TE (=48.7ms) above the level of noise?

We used TE’s=6.3-32 ms

Greater detail of the Pimonidazole staining protocol (or reference to appropriate paper/protocol) is required.

Pimonidazole was administered via intraperitoneal injection. The rabbits were killed after 1.5-hour pimonidazole (60 mg/kg) administration. Perfusion (3% paraformaldehyde) fixed kidneys were cut into 4-µm sections and processed for immunostaining with Dako EnVision System-HRP kit (Carpinteria, CA) according to manufacturer’s procedure. Briefly, after deparaffinization, antigen retrieval and peroxidase quenching, sections were incubated with protein blocking solution for 20 minutes. The sections were sequentially incubated with a specific rabbit antipimonidazole antibody diluted 1:100 (Natural Pharmacia Inc) for 1 hour, and labeled with polymer-HRP antirabbit secondary antibody. Pimonidazole staining was visualized with DAB chromogen, followed by counterstaining with hematoxylin. Renal hypoxia was evaluated by semi-quantitative determination of pimonidazole staining of tubules in the cortex and medulla.

Results:
Justify with all BOLD images were deemed “high-quality”. For example was this justified by the decision to include TE’s in T2* map construction (as mentioned above)?

Surely the high quality standard is to rely on individual subjective and not have the single standard. In order to avoid misunderstanding, we have delete the “high-quality”.

Data for the control animals are not given in tables 1 and 2. These should be included to demonstrate the effectiveness of inducing diabetes.

The reviewer is correct and we have added the control group in tables 1 and 2.

Table 4 should include a control group. The authors make it clear that no R2* differences are observed between the type I and type II diabetes rabbits following 350mgI/ml of contrast but do not provide important statistical comparisons with controls. These data would be valuable to demonstrate whether the contrast agent had any effect on the kidney independent of diabetes.

The reviewer is correct and we have added the control group in table 4.

Figure 1: error bars should be included on all R2* measurements

We have added the error bars.

Figures 2, 3 & 4: inclusion of images from a control group would greatly improve these figures.

We have added the control group figures in fig 2, 3, 4.

Figure 4: Does the pimonidazole only stain the outer medulla as the images suggest? This immunohistochemical method becomes insensitive at low pO2, an accepted limitation of the method. Lower magnification images should be shown to demonstrate the full extent of the immunopositive staining in the kidney.

Both of the cortex and outer medulla have stain, but outer medulla dyeing is obvious. We used the reference (Prasad PV. Evaluation of intra-renal oxygenation by BOLD MRI. Nephron Clin Pract 2006; 103: c58-65.) to support our methods.

Discussion:
The question of whether iodine based contrast agents can alter the BOLD signal is a sound clinical concern which has been previously explored by others (e.g. Heneder et al. Invest Radiol. 2012 May;47(5):299-305). It should be made clear by the authors how the present study builds on this work.

Response:
First, in the study of Haneder et al. (now ref. 13) they used a Siemens 1.5T MR scanner, but we used a GE 3.0T MR scanner. The results of Li et al. show that the different field intensities of MR scanners can affect the R2* values (Li LP et al. Evaluation of intrarenal oxygenation by BOLD MRI at 3.0 T. J Magn Reson Imaging 2004; 20:901-904.). Prasad et al. also suggested that measurements of R* values at 3.0 T are preferred when a choice is available. (Prasad PV et al. Evaluation of intra-renal oxygenation by BOLD MRI. Nephron Clin Pract. 2006; 103(2): c58-65).

Second, the experimental subjects are different. Haneder et al. used pigs as the subjects in their study, but we used rabbits as the experimental subjects. R2* values also depend on other factors, such as homogeneity of the tissue (Simon-Zoula SC et al. Non-invasive monitoring of renal oxygenation using BOLD-MRI: a reproducibility study. NMR Biomed. 2006; 19(1): 84-9).

Third, the experimental process of the study of Haneder et al. is different from ours; they injected the 300 contrast medium into the same pig at day 1 and as the 320 contrast medium at day 2. Whether the administration of contrast agent would result in accumulations of iodine content and viscosity and further influence the accuracy of R2* values is not discussed in the literature.

Fourth, Haneder et al. used two different contrast agents, separately supplied by the Bayer Company and GE Company. We used four kinds of contrast media, all supplied by the GE Company in our research, which provides for a good degree of consistency.

Fifth, if the contrast medium has paramagnetic properties, and it is known that it remains in the kidneys for several hours after injection and affect the MR values, then any effect on measurements would be greatest 1 to 55 minutes after injection, but this is not shown in the study of Haneder et al. After one hour of circulation and metabolism through the body (with different heart rates: 258 ± 2.8 beats/min for rabbits and 80-90 beats/min for pigs), the contrast agent is excreted from the kidneys. Therefore, the potential paramagnetic property of the contrast agent would not seriously affect the R2* values from 1 h to 72 h in our research, especially from 24 h to 48 h after injection of contrast medium.

Limitations:
As mentioned, the dose-finding experiment on the type II diabetics does not reach sufficiently high doses to suggest maximum signal by the 350 dose. Similarly, application of this dose to type I diabetics was performed without justification – could the type II and II diabetic animals respond differently to higher iodine contrast doses?

We have changed the parts in the manuscripts.

Reproducibility of BOLD scans. The authors do not indicate in the methods whether BOLD scans
were repeated for each time point. All scans should be repeated in triplicate to explore stability, if this data exists it should be included in the manuscript. Otherwise, the errors given for multiple ROI on a single scan at each time point should be mentioned as a limitation.

To objective of the study, we have added the repeat BOLD scans for each time point and fixed the ROI. The ROI was set to 5 mm$^3$ (containing at least 30 pixels).

- **Minor Essential Revisions**
  
  *Note: spelling and grammar errors can be referred to once the authors provide a line numbered manuscript.
  
  For clarity change “in a model related to rabbits’ diabetic nephropathy…” to “a rabbit model of diabetic nephropathy”.

  **Change made as indicated by the reviewer.**

**Abstract:**

Clarify that rabbits were indeed first treated with different compounds to induce type I or II diabetes. Currently the suggestion is that rabbits were already predisposed to diabetes.

“Besides, the 350 treatment group…” The use of the word “besides” is unnecessary here and in many other parts of the manuscript (these can be addressed when the authors provide a line numbered manuscript).

  **Change made as indicated by the reviewer.**

**Methods:**

Change “glycemic index value was over 100…” to “glycemic index value was over 100…”

  **Change made as indicated by the reviewer.**

**Results:**

The authors state that each scan was “measured independently in triplicate by three professional radiologists”. The independent analysis by three radiographers adds a robust element to this paper. This is punctuated by the ongoing debate into the validity of BOLD MRI in the kidney (e.g. Inoue et al. Kidney International (2012) 82, 934). Inclusion of the individual results found by each radiographer would therefore provide a valuable addition to the dataset and support the on-going challenges of the wider BOLD MRI community in finding standardized measurement procedures. Other approaches to standardise BOLD analysis have been proposed by others and these might be worthy of comment. For example: 1) Ebrahimi et al. Invest Radiol. 2012 Mar;47(3):175-82 2) Menzies et al. AJP Renal Physiol 305: F845–F852, 2013. Figure 3: injury scoring (allowing for a quantitative statistical analysis) would be a valuable addition to the histological analysis including a control group.

  **Change made as indicated by the reviewer.**

- **Discretionary Revisions**

  Consider changing “contrast agent” throughout the manuscript to “iodine contrast agent (ICA)” or equivalent to make it clear to the reader that the iodine concentration, not contrast agents in general, is being investigated here.

  **Level of interest:** An article whose findings are important to those with closely related research interests

  **Quality of written English:** Not suitable for publication unless extensively edited

  **Statistical review:** No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report
Title: Effects of iodinated contrast agents on renal oxygen level by BOLD MRI in a model related to rabbits' diabetic nephropathy
Version: 6 Date: 3 April 2014
Reviewer: Matthew Bailey

Major Compulsory Revisions

1. The objective of the study is not clear and the authors must clarify the questions that they are asking. This is a large study and the data generated are of interest. It is difficult to understand if the authors are assessing the effect of diabetes on the intrarenal distribution of $R_2^*$ or the effect of contrast nephropathy. The interaction between the two is interesting but the paper lacks fundamental characterization of either insult.

   The objective of the study has been changed to be more explicit and now appears as follows in discussion parts:

   Under physiological conditions, $\text{PaO}_2$ in renal tissue is low, especially in the outer medulla. Palm et al. found that oxygen consumption of the renal medulla was higher in diabetes mellitus because metabolic activity was increased. Furthermore, a study found that the kidney of diabetic rats consumed about 40% more oxygen, which was related mainly to high metabolic activity when compared to nondiabetic rats. Consequently, it was concluded that hypoxia is associated with the development of diabetic nephropathy despite controlled glycemia. In diabetic nephropathy, because of increased $\text{Na}^+\text{-K}^+\text{-ATP}$ enzyme activity and overloaded kidney tubules, especially in the proximal convoluted tubule and thick ascending limb located in the outer medulla, there was high oxygen consumption, so that renal function suffered further deterioration. In addition, glomerular hyperfiltration led to increased oxygen consumption. This was confirmed by our findings that the $R_2^*$ values of the renal outer medulla were higher than other parts of the kidney in the type II diabetic nephropathy group, indicating that the renal outer medulla had lower oxygen saturation.

   After different iodinated contrast agents were injected, direct nephrotoxicity damaged glomerular cells by increasing permeability, and led to apoptosis of the tubular cells. Because of the viscosity of iodinated contrast medium, the arterially-supplied oxygen was reduced. Meanwhile, the small renal vessels tended to be blocked easily as the viscosity was increased. Iodinated contrast agents seem to diminish renal blood flow by increasing plasma viscosity. These findings might be explained by decreased renal perfusion or higher oxygen consumption, which would lead to decreased local oxygen content, especially in the renal outer medulla.

2. There is no control group for the diabetes (either type 1 or type 2). Without this, the data are difficult to interpret in the appropriate context. The authors indicate what they are testing two distinct models of diabetic nephropathy but how is this confirmed in the absence of a control group of rabbits? Some histological evidence of DN should be provided to assess the extent of injury between the type 1 and type 2 models and against a control.

   We have added the control group in the experiment.

3. What are the effects of contrast agents on renal $R_2^*$ in control rabbits? Without this, the comparison between type 1 and 2 is difficult to interpret in a meaningful way.

   We have added the control group in the experiment.
4. I agree that CIN is an important clinical problem. The authors give example images in Fig 4 for the highest dose given to a rabbit with type 2 diabetes. However, there are no data that allow a quantitative comparison of CIN across doses in the type 1 and type 2 rabbits. A blinded scoring of the histology is required in all groups so that the BOLD data can be interpreted by relating to the extent of CIN.

Thank you for your advice. The reviewer is correct and we would like to add the blinded scoring of the histology in my manuscript. We would like to add the blinded scoring of the histology as soon as possible. Although didn't mention in the article, we have completed a survey of the quantitative comparison of CIN across doses in the type 1 and type 2 rabbits. Please give me a chance to review my manuscript again, if the manuscript does not have other questions, we would to provide relevant information immediately.

5. It is good that the authors have used the pimonidazole-adduct IHC approach as an independent measurement of "oxygenation". A quantitative assessment of this is required in addition to the exemplar figures shown.

We have added the quantitative assessment in the results.

Minor Essential Revisions
1. The manuscript is difficult to read in places and the overall objective of the study is difficult to follow. There are numerous small errors throughout and I recommend that the authors seek some proof-reading help.

We have changed the grammatical errors in this manuscript and provided the manuscript with page/line numbers.

2. The discussion is superficial at the moment but if the authors can relate the changes in the BOLD MRI signal to the underlying pathology, this will add significant depth. Similarly, the authors should be able to relate the information from MRI with the less sensitive pimonidazole-adduct IHC, to discuss better the implications of diabetes/CIN for intrarenal oxygenation.

We have changed the discussion parts in order to clear the related changes in the BOLD MRI signal to the underlying pathology.

3. The authors need to provide more detail for the pimonidazole approach- when was the probe injected, how were the rabbits killed and how long after were the kidneys removed.

Pimonidazole was administered via intraperitoneal injection. The rabbits were killed after 1.5-hour pimonidazole (60 mg/kg) administration. Perfusion (3% paraformaldehyde) fixed kidneys were cut into 4-μm sections and processed for immunostaining with Dako EnVision System-HRP kit (Carpinteria, CA) according to manufacturer’s procedure. Briefly, after deparaffinization, antigen retrieval and peroxidase quenching, sections were incubated with protein blocking solution for 20 minutes. The sections were sequentially incubated with a specific rabbit antipimonidazole antibody diluted 1:100 (Natural Pharmacia Inc) for 1 hour, and labeled with polymer-HRP antirabbit secondary antibody. Pimonidazole staining was visualized with DAB chromogen, followed by counterstaining with hematoxylin. Renal hypoxia was evaluated by semi-quantitative determination of pimonidazole staining of tubules in the cortex and medulla.

Level of interest: An article of limited interest
Quality of written English: Needs some language corrections before being published
Statistical review: Yes, but I do not feel adequately qualified to assess the
statistics.

Declaration of competing interests:
I declare that I have no competing interests