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TEMPOL has limited protective effects on renal oxygenation and hemodynamics but reduces kidney damage and inflammation in a rat model of renal ischemia/reperfusion by aortic clamping

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Dear Dr. Ergin,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. The reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Your revision is due by Aug 31, 2015.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: Referee report for JCTRES-S-15-00006
Ergin et al. used the superoxide scavenger TEMPOL to ameliorate renal damage in a clinically relevant rat model of aortic cross clamping-induced kidney ischemia/reperfusion (I/R) injury. The main conclusion is that although TEMPOL reduced inflammation in the reperfused kidney, more effective intervention modalities are needed to also counteract the severe hemodynamic perturbations that are inherent to prolonged aortic occlusion.

1. General commentary
Strengths:
* The study investigates an important clinical problem in an appropriate preclinical I/R model and as such fits the journal's scope.
* The paper entails some elegant experimental techniques (e.g., OxyphorG2 phosphorescence lifetime measurements) to assess post-ischemic renal physiology and therefore holds considerable news value.
* The authors are realistic about the efficacy of their intervention with respect to the most relevant outcome parameters.

Weaknesses:
* The choice of analytical techniques is not always aligned with the experimental hypotheses and results discussed in the paper (e.g., lack of kidney histology, plasma creatinin levels were not measured). Authors are therefore forced to largely speculate about the mechanistic underpinnings of their findings. While this is adequately done and does not preclude publication of the work, the authors should always phrase their speculations with caution. Additionally, the discussion would benefit from a short 'critique of methods' section.
* The JCTR guidelines stipulate that the length of the manuscript must be proportional to the conveyed message. In view of the relatively modest number of display items, the manuscript could perhaps be shortened without compromising the quality of the paper.

2. Introduction
* On page 4, lines 16-17, it is mentioned that aortic aneurysm repair in kidney transplant patients is an important cause of AKI. It is more logical to generalize this statement to all patients who require surgical repair of an aortic aneurysm, as this is a far more prevalent etiology.
* On page 4, lines 26-28, NO is mentioned as reactive nitrogen species that "contributes to microvascular dysfunction and debilitated microvascular oxygenation in I/R-subjected kidneys". Although NO has an unpaired electron and therefore is indeed a radical, the main biological function of NO is vasodilatation, which predominantly reduces I/R injury in most preclinical models. Please rephrase or explain why NO is actually harmful in AKI/kidney I/R.
* On page 5, lines 40 - 41, the role of endothelial cell NADPH oxidase in oxidative stress following kidney I/R is mentioned. Could the authors indicate which of the endothelial NOX isoforms is/are expected to contribute to post-ischemic kidney injury? This is relevant as the

endothelial NOX isoforms differ with respect to superoxide-generating capacity and subcellular localization (e.g., see PMID 15706079). The latter in turn affects the type of injury caused by enzyme overactivation.
* On page 5, lines 47 - 48 "Superoxide also reacts with nitric oxide (NO)". The reaction between NO and superoxide operates at a diffusion-controlled rate and therefore predominates over virtually all other competing reactions. Please add this nuance to the aforementioned passage. Also, nitric oxide was already abbreviated earlier in the introduction.
* The role of peroxynitrite is elaborately discussed on page 5 and 6. Have nitration footprints (e.g., 3-nitrotyrosines) ever been found in clinical kidney I/R samples? That would further strengthen the importance of detoxifying superoxide with, e.g., TEMPOL.

3. Materials and methods
* The anesthetic regimen is described on page 8. As it is claimed that all animal experiments were performed in accord with most recent ethical guidelines, I assume this combination of compounds also provides analgesia?
* When possible, the solvent and solvent concentrations used to administer, e.g., TEMPOL and Oxyphor G2 should be added to the manuscript, particularly since administering large volumes of solvents such as DMSO can affect both hemodynamic as well as oxidative stress readouts.
* Considering animal hemodynamics are an instrumental parameter in the presented experiments, could the authors briefly comment on their fluid management?
* The figure on page 9 is erroneously labeled as Figure 1 (should be Figure 2).
* The experimental protocol is described in section 2.4 on page 10. First, the sham operation should be described in more detail, especially considering (the trend towards a) decrease in mean arterial pressure in control animals shown in Table 1. Is an aortic clamp also place (but not closed) during sham surgery? Was the left kidney also exposed and decapsulated? This also relates to the second question, which is whether the authors are sure the blood supply to (or innervation of) the adrenal glands was spared during these operations. Adrenal injury would, e.g., explain the drop in blood pressure seen in all experimental groups as a result of inadequate aldosterone signaling. Third, why did the authors elect to use TEMPOL, instead of the pharmacokinetically more favorable mitochondria-targeted analogue MitoTEMPO? Last, are the pharmacokinetic properties of intravenously administered TEMPOL at the used dose adequate to attenuate kidney I/R (i.e., is a sufficient tissue concentration reached?)?
* On page 11, line 1, it is mentioned that Oxophor G2 binds to albumin and therefore is retained in the vasculature. However, a large part of the discussion is devoted to the effects of hind limb I/R on hemodynamics. If the drop in blood pressure is indeed attributable to hind limb I/R, this can be explained by either a systemic (i.e., sepsis-like) or local capillary leak. In both scenarios, albumin translocates to the extracellular compartment, thereby (partially) explaining the hypotensive episode. As delivery of Oxyphor G2 apparently relies on albumin, is phosphorescence lifetime imaging possible in experimental models with anticipated hypoalbuminemia?
* As it is important to disseminate information on the correct techniques to measure oxidative stress parameters, the section on mass spectrometric analysis of MDA should be accompanied by a reference to a recent editorial published in Free Radical Biology and Medicine (PMID 25462642).

4. Results and discussion

* On page 15, line 17, "the degree of iNOS activation" should be changed to for instance "iNOS expression" as immunohistochemistry cannot measure enzyme activation.
* Please remove the passage between parentheses on page 15, lines 41-44, to improve legibility.
* Plasma IL-6 levels are mentioned on page 16, line 1. However, the materials and methods section only mentions IL-6 immunohistochemistry. Please clarify.
* Page 16, lines 7-13 ("The same … Figure 3A and B") highlight that TEMPOL reduced iNOS expression, IL-6 production, and NGAL staining in control animals. The fact that TEMPOL is biologically active in absence of I/R should be briefly discussed. Does this mean that the sham operation (or maybe other experimental factors?) already induces inflammatory signaling without an ischemic hit?
* The role of iNOS activation and NO bioavailability is discussed on page 16, lines 14 - 44. The first finding is that TEMPOL reduced iNOS expression following I/R, probably by reducing superoxide-induced inflammatory signaling. The fact that this implies TEMPOL treatment actually reduced NO production should be briefly discussed, especially in light of the improved RVR conferred by TEMPOL (Table 1). The results generated in reference [55] support the premise that TEMPOL decreases iNOS-mediated bursts in NO production, but improves net NO bioavailability via superoxide scavenging and preventing oxidative stress-induced eNOS uncoupling. The discussion about the effect of the TEMPOL-mediated reduction in iNOS staining on the one hand and the hemodynamic consequences of altered NO bioavailability on the other hand should be simplified (see for instance PMID 9648722).
* On page 17, lines 17 - 28, "acid/base excess" should be changed to "base excess". Also, it may be helpful to include the complete blood gas results in the main manuscript, which would enable the reader to assess the state and severity of the I/R-induced metabolic acidosis.
* On page 18, lines 7 - 10 "Where sufficient … reperfusion phase [10]" contradict the statement on the previous page that peroxynitrite is formed "during all phases of I/R".
* The subcellular effects of antioxidant enzymes are discussed on page 19, lines 31 - 49. It is postulated that the hydrophilic nature of TEMPOL precludes a protective effect on lipid peroxidation. However, the authors next indicate that TEMPOL prevents the formation of tertiary species by detoxifying superoxide. Wouldn't this also reduce lipid peroxidation mediated by these tertiary derivatives of superoxide?
* Renal lactate metabolism is addressed on page 19 to interpret the metabolic acidosis invoked by aortic cross clamping. Although regulation of renal lactate processing is elaborately discussed, the discussion could be geared more towards the presented experimental findings. A contribution of renal to lactate metabolism in the performed experiments requires two factors that the authors claim are critically reduced in their model: renal mitochondrial metabolism (discussed on page 25-26) and glomerular filtration. Normally, metabolic acidosis during AKI reflects a defective clearance of acids and an impaired production of HCO3-. In addition, the mean arterial pressure <40 mmHG indicates a severe hypotensive episode during which all organs and not just the kidneys produce lactate as a result of anaerobic respiration. Last, the authors should address in more detail how TEMPOL was able to influence acid/base disturbance (pH, lactate) following I/R without affecting mean arterial pressure or kidney oxygenation.
* On page 20, lines 53 - 54, it noted that 'moderate oxidative stress' was observed in the current experiments. It seems to me that immunohistochemistry is unable to distinguish between intracellular and extracellular (i.e., released) MPO. Also, the authors did not measure

H2O2 levels, which is required for MPO to produce hypohalous acids and induce oxidative injury. Statements on oxidative stress on the basis of MPO staining only are therefore inappropriate.
* The plea for using the right animal model made on page 21, lines 3 - 6 is commendable, but the model actually induced (irreversible) anuria and a mean arterial pressure of <40 mmHG. This strikes me as a severe and potentially lethal injury model and not as "moderately injurious" (page 21, lines 43-45). Please comment.
* Page 22, lines 12 - 25 ("Illustratively … artery occlusion") can be summarized or omitted to improve legibility.
* As briefly mentioned above, the drop in blood pressure (Table 1) in the control group is worrying should be addressed in the discussion.
* The data in Table 1 and Table 2 indicate an I/R-induced reduction in renal perfusion and oxygen delivery. Normally, the juxtaglomerular apparatus would respond to such alterations by upregulating the renin-aldosterone axis in order to increase renal perfusion by increasing systemic blood pressure. Can the authors comment on the apparent failure of this compensatory mechanism?
* The last section of manuscript (page 23 - 27) deals with the effects of I/R and TEMPOL treatment on various renal oxygenation parameters. The lines of reasoning in this section in general seem very sound. As the critically low mean arterial pressure explains the reduction in most measured oxygenation parameters, the authors highlight the unaltered renal oxygen extraction rate (O2ER) as most striking finding. It light of this finding, it should be noted that kidneys are stable to maintain a stable O2ER over a wide range of conditions (PMID 14416431). However, an increase in renal O2ER has also been reported in clinical post-ischemic AKI (PMID 23514538), albeit under diuresis and related renal oxygen consumption was still intact in these experiments. Please comment on these findings in relation to the data shown in Table 2.
* It is mentioned on page 26, line 6-9 that "it seems that the metabolic demands of renal cells were met". How does this statement fit with the marked drop in medullary and cortical microvascular oxygen tensions seen in I/R-exposed rats?
* When comparing the current results to the data from ref [55], a large contribution of aortic cross clamping to the drop in mean arterial pressure is plausible. The authors repeatedly emphasize the expected consequences of hindleg I/R on blood pressure. As this is an important mechanistic explanation for the presented findings, the (potential) factors via which hind limb I/R suppressed the mean arterial pressure should be discussed in more detail.
* Although statistical significance is not reached, nearly all hemodynamic and oxygenation parameters included in Table 1 and Table 2 show a trend towards improvement in TEMPOL-treated animals. Have the authors considered using a higher TEMPOL dose? Do they expect this would yield better results with respect to clinically relevant end point (i.e., kidney function/diuresis?).

Reviewer #2: The study on the protective effect of TEMPOL on I/R in rats is well conducted, well written. No surprise with the limited protective effect because ischemia reperfusion may induce tissue injury by several pathways and sterile inflammation and oxidative stress are among many other pathways. As the authors themselves state a superoxide scavenger as TEMPOL has some meliorative effect, but per se is probably not enough and should be

associated with other drugs or strategies. Translating these results in renal transplantation is even more hazardous because in organ transplantation in addition to arterial clamping we do have other effects as the stimulation of innate and adaptive immunity. This latter point should be discussed and better clarified by the authors

Reviewer #3: This study was conducted in order to evaluate the renoprotective effects of tempol against aortic occlusion-induced renal ischemia/reperfusion and the following renal damage.

The pre- and post-ischemic treatment with tempol at a cumulative dose of 350 mmol/kg produced a protective effect against renal damage, inflammation, and iNOS expression, although the beneficial effects on renal hemodynamics and oxidative stress.

The purpose of the study is clear, and methodology is also very well, findings are well-discussed.

I have only one minor comment, as follows: The rationale of the dose of tempol should be described in the method.

In addition, the photograph of surgery is "Figure 2".

Author's response

Reviewer 1:

We thank the reviewer for his/her comments.

On page 4, lines 16-17, it is mentioned that aortic aneurysm repair in kidney transplant patients is an important cause of AKI. It is more logical to generalize this statement to all patients who require surgical repair of an aortic aneurysm, as this is a far more prevalent etiology.

Answer: we changed statement to "the most common cause of AKI in the clinical setting is suprarenal aortic clamping [4], which is performed in procedures such as kidney transplantation [5], pancreas transplantation [6], and aortic aneurysm repair [7]."

On page 4, lines 26-28, NO is mentioned as reactive nitrogen species that "contributes to microvascular dysfunction and debilitated microvascular oxygenation in I/R-subjected kidneys". Although NO has an unpaired electron and therefore is indeed a radical, the main biological function of NO is vasodilatation, which predominantly reduces I/R injury in most preclinical models. Please rephrase or explain why NO is actually harmful in AKI/kidney I/R.

Answer: There are many studies indicating that the levels of the three different NO subtypes vary under different pathological conditions. Under pathological conditions such as I/R, the inducible form of NO is most predominant, and high level of iNOS-

derived NO lead to impaired tissue and organ function as a result of oxidative/nitrosative stress.

On page 5, lines 40 - 41, the role of endothelial cell NADPH oxidase in oxidative stress following kidney I/R is mentioned. Could the authors indicate which of the endothelial NOX isoforms is/are expected to contribute to post-ischemic kidney injury? This is relevant as the endothelial NOX isoforms differ with respect to superoxide-generating capacity and subcellular localization (e.g., see PMID 15706079). The latter in turn affects the type of injury caused by enzyme overactivation.

Answer: The isoforms have been specified in the text and properly referenced.

On page 5, lines 47 - 48 "Superoxide also reacts with nitric oxide (NO)". The reaction between NO and superoxide operates at a diffusion-controlled rate and therefore predominates over virtually all other competing reactions. Please add this nuance to the aforementioned passage. Also, nitric oxide was already abbreviated earlier in the introduction.

Answer: Excellent point, which has been implemented in the text.

The role of peroxynitrite is elaborately discussed on page 5 and 6. Have nitration footprints (e.g., 3-nitrotyrosines) ever been found in clinical kidney I/R samples? That would further strengthen the importance of detoxifying superoxide with, e.g., TEMPOL.

Answer: Thiol-containing agents such as glutathione, albumin, and cysteine are able to convert peroxynitrite to nitrosothiols as a metabolite, demonstrating antineutrophil and cardioprotective properties [PMID 10615389]. However, detection of the nitrosothiols in biological samples is difficult. A PubMed search using “3-nitrotyrosines renal ischemia reperfusion” yielded 0 results.

The anesthetic regimen is described on page 8. As it is claimed that all animal experiments were performed in accord with most recent ethical guidelines, I assume this combination of compounds also provides analgesia?

Answer: In addition to the ketamine and dexmedatomidine that were used for anesthesia and sedation at the time of induction, ketamine was continuously administrated throughout entire experiment to provide analgesic care [PMID 26283834].

When possible, the solvent and solvent concentrations used to administer, e.g., TEMPOL and Oxyphor G2 should be added to the manuscript, particularly since administering large volumes of solvents such as DMSO can affect both hemodynamic as well as oxidative stress readouts.

Answer: we dissolved these compounds in saline and specified this in the text.

Considering animal hemodynamics are an instrumental parameter in the presented experiments, could the authors briefly comment on their fluid management?

Answer: Fluid management was performed as specified in the text:

"The right jugular vein was cannulated for continuous infusion of Ringer's lactate (Baxter Healthcare, Deerfield, IL) at a rate of 15 mL/kg/h and of maintenance anesthesia (50 mg/kg/h ketamine dissolved in Ringer's lactate, 5 mL/kg/h)."

The figure on page 9 is erroneously labeled as Figure 1 (should be Figure 2).

Answer: it now reads "Figure 2," thank you.

The experimental protocol is described in section 2.4 on page 10. First, the sham operation should be described in more detail, especially considering (the trend towards a) decrease in mean arterial pressure in control animals shown in Table 1. Is an aortic clamp also place (but not closed) during sham surgery? Was the left kidney also exposed and decapsulated? This also relates to the second question, which is whether the authors are sure the blood supply to (or innervation of) the adrenal glands was spared during these operations. Adrenal injury would, e.g., explain the drop in blood pressure seen in all experimental groups as a result of inadequate aldosterone signaling. Third, why did the authors elect to use TEMPOL, instead of the pharmacokinetically more favorable mitochondria-targeted analogue MitoTEMPO? Last, are the pharmacokinetic properties of intravenously administered TEMPOL at the used dose adequate to attenuate kidney I/R (i.e., is a sufficient tissue concentration reached?)?

Answer: 1. Aortic clamping was not employed in sham groups but all surgical procedures such as tracheotomy, cannulations, renal decapsulation, separation of renal pedicles, and the placement of the measurement devices were exactly the same for each group. To clarify this, the following text was added:

"It should be noted that sham-operated animals underwent the same procedures as described in sections 2.2, 2.3, and above except for the cross-clamping of the aorta."

2. We were not concerned about safeguarding the blood supply to the adrenal gland because decapsulation of the kidney concurs with removal of the adrenal gland. Indeed, this may have perturbed the renin/angiotensin system, but the reviewer should note that only one kidney was decapsulated. Residual renin/angiotensin functionality remained as a result of the intact second kidney. Moreover, in a previous study from our group (Aksu et al., Intensive Care Med Exp 2015;3:21) the same procedure was performed without causing a decline in MAP, pleading against the phenomenon implied by the reviewer.

3. In our earlier study we had tested the effect of TEMPOL on renal arterial clamping-induced renal I/R injury. In this follow-up study we induced acute kidney injury by aortic clamping rather than renal artery clamping, which is a more clinical relevant approach.

4. This dose of the TEMPOL had been validated in the previous study (Aksu et al., Intensive Care Med Exp 2015;3:21). It is, however, possible that for this injury model it was insufficient. Accordingly, the last sentence of the Discussion reads:

"However, additional studies are warranted to examine the efficacy of higher TEMPOL doses and the pathological contribution of separate anatomical compartments to post-ischemic kidney injury."

On page 11, line 1, it is mentioned that Oxophor G2 binds to albumin and therefore is retained in the vasculature. However, a large part of the discussion is devoted to the effects of hind limb I/R on hemodynamics. If the drop in blood pressure is indeed attributable to hind limb I/R, this can be explained by either a systemic (i.e., sepsis-like) or local capillary leak. In both scenarios, albumin translocates to the extracellular compartment, thereby (partially) explaining the hypotensive episode. As delivery of Oxyphor G2 apparently relies on albumin, is phosphorescence lifetime imaging possible in experimental models with anticipated hypoalbuminemia?

Answer: The potential ramifications of hypoalbuminemia are not expected to impair measurements because hypoalbuminemia and corollary lower Oxyphor G2 concentration in blood would only decrease the signal amplitude but would not affect the phosphorescence lifetime, unless the extravascular space had a significantly lower pO2 than the vasculature. In our model, however, this is not very likely.

As it is important to disseminate information on the correct techniques to measure oxidative stress parameters, the section on mass spectrometric analysis of MDA should be accompanied by a reference to a recent editorial published in Free Radical Biology and Medicine (PMID 25462642).

Answer: The proper references have been included in the text.

On page 15, line 17, "the degree of iNOS activation" should be changed to for instance "iNOS expression" as immunohistochemistry cannot measure enzyme activation.

Answer: Good point; this has been changed throughout the text.

Please remove the passage between parentheses on page 15, lines 41-44, to improve legibility.

Answer: The passage has been removed.

Plasma IL-6 levels are mentioned on page 16, line 1. However, the materials and methods section only mentions IL-6 immunohistochemistry. Please clarify.

Answer: This has been changed to “This trend was mirrored by increased IL-6 immunostaining (Figure 3B)…”

Page 16, lines 7-13 ("The same … Figure 3A and B") highlight that TEMPOL reduced iNOS expression, IL-6 production, and NGAL staining in control animals. The fact that TEMPOL is biologically active in absence of I/R should be briefly discussed. Does this mean that the sham operation (or maybe other experimental factors?) already induces inflammatory signaling without an ischemic hit?

Answer: Yes. We have modified the respective sentence to “The same trend was observed in

TEMPOL-treated rats that did not undergo I/R compared to baseline levels (Ctrl group, Figure 3A and B), most likely because the surgical procedures alone lead to a mild sterile immune response."

On page 17, lines 17 - 28, "acid/base excess" should be changed to "base excess". Also, it may be helpful to include the complete blood gas results in the main manuscript, which would enable the reader to assess the state and severity of the I/R-induced metabolic acidosis.

Answer: The phrasing now reads "base excess." The pH data are provided in the supplemental information. Other relevant information is provided in Fig. 3F and I.

Renal lactate metabolism is addressed on page 19 to interpret the metabolic acidosis invoked by aortic cross clamping. Although regulation of renal lactate processing is elaborately discussed, the discussion could be geared more towards the presented experimental findings. A contribution of renal to lactate metabolism in the performed experiments requires two factors that the authors claim are critically reduced in their model: renal mitochondrial metabolism
(discussed on page 25-26) and glomerular filtration. Normally, metabolic acidosis during AKI reflects a defective clearance of acids and an impaired production of HCO3-. In addition, the mean arterial pressure <40 mmHG indicates a severe hypotensive episode during which all organs and not just the kidneys produce lactate as a result of anaerobic respiration. Last, the authors should address in more detail how TEMPOL was able to influence acid/base disturbance (pH, lactate) following I/R without affecting mean arterial pressure or kidney oxygenation.

Answer: We added the following text to the respective section:

"Renal contribution to lactate metabolism is influenced by renal mitochondrial metabolism (which seemed to be unperturbed based on the O_2ER_{ren} data) and glomerular filtration (which was absent). The lactate data may therefore also have been skewed by mainly the fact that the I/R-subjected kidneys were hypoperfused (section 3.2.1) and anuric in all groups (data not shown)."

The plea for using the right animal model made on page 21, lines 3 - 6 is commendable, but the model actually induced (irreversible) anuria and a mean arterial pressure of <40 mmHG. This strikes me as a severe and potentially lethal injury model and not as "moderately injurious" (page 21, lines 43-45). Please comment.

Answer: The reviewer should note that we used the phrasing "moderately injurious ischemia time" in reference to longer durations of ischemia implemented in other studies as well as our data. Although the 30-min ischemia time is moderately injurious to the kidney itself, the systemic effects apparently predominate (i.e., the central message of this paper) and account for the severity of this model.

Page 22, lines 12 - 25 ("Illustratively … artery occlusion") can be summarized or omitted to improve legibility.

Answer: In this section we provide a direct comparison to the earlier study to underscore the profound differences in hemodynamics induced by aortic clamping versus renal

artery clamping. We feel this is part of the essence of our study and therefore prefer to keep this section intact.

As briefly mentioned above, the drop in blood pressure (Table 1) in the control group is worrying should be addressed in the discussion.

Answer: We added the following text (underlined) to address this concern:

"Illustratively, the MAP in this study decreased by 38% and 47% at R15 and R90, respectively, versus a decrease of merely 7% and −2%, respectively, in case of renal artery clamping [58]. These data might be explained by the suprarenal aortic occlusion, which also blocks the inferior mesenteric artery blood supply and may lead to sepsis-induced hypotension due to increased bacterial translocation into blood stream."

The data in Table 1 and Table 2 indicate an I/R-induced reduction in renal perfusion and oxygen delivery. Normally, the juxtaglomerular apparatus would respond to such alterations by upregulating the renin-aldosterone axis in order to increase renal perfusion by increasing systemic blood pressure. Can the authors comment on the apparent failure of this compensatory mechanism?

Answer: This is an astute comment. The juxtaglomerular apparatus normally responds to alterations in renal perfusion by upregulating the RAS in order to increase renal perfusion by increasing systemic blood pressure. In our model, the deteriorating effect of I/R on the kidney as well as systemic parameters may have impaired the renin-angiotensin-aldosterone signaling axis.

The last section of manuscript (page 23 - 27) deals with the effects of I/R and TEMPOL treatment on various renal oxygenation parameters. The lines of reasoning in this section in general seem very sound. As the critically low mean arterial pressure explains the reduction in most measured oxygenation parameters, the authors highlight the unaltered renal oxygen extraction rate (O2ER) as most striking finding. It light of this finding, it should be noted that kidneys are stable to maintain a stable O2ER over a wide range of conditions (PMID 14416431). However, an increase in renal O2ER has also been reported in clinical post-ischemic AKI (PMID 23514538), albeit under diuresis and related renal oxygen consumption was still intact in these experiments. Please comment on these findings in relation to the data shown in Table 2.

Answer: ERO2 is dependent on the balance between oxygen delivery and consumption, which is determined by cell metabolism, Na/K ATPase function, and ROS production. In this study we showed that TEMPOL treatment improved DO2 and VO2. Accordingly, we suggest that TEMPOL treatment increased the bioavailability of oxygen, thereby supporting the kidney's metabolic needs.

In line with your useful comments we did add the following sentence to the respective paragraph:

"In that respect, it has been reported that kidneys are quite able to maintain a stable O_2ER over a wide range of conditions [108]."

Although statistical significance is not reached, nearly all hemodynamic and oxygenation parameters included in Table 1 and Table 2 show a trend towards improvement in TEMPOLtreated animals. Have the authors considered using a higher TEMPOL dose? Do they expect this would yield better results with respect to clinically relevant end point (i.e., kidney function/diuresis?).

Answer: Yes, we have. This is illustrated in the last sentence of the Discussion:

"However, additional studies are warranted to examine the efficacy of higher TEMPOL doses and the pathological contribution of separate anatomical compartments to post-ischemic kidney injury."

Reviewer 2:

The study on the protective effect of TEMPOL on I/R in rats is well conducted, well written. No surprise with the limited protective effect because ischemia reperfusion may induce tissue injury by several pathways and sterile inflammation and oxidative stress are among many other pathways. As the authors themselves state a superoxide scavenger as TEMPOL has some meliorative effect, but per se is probably not enough and should be associated with other drugs or strategies. Translating these results in renal transplantation is even more hazardous because in organ transplantation in addition to arterial clamping we do have other effects as the stimulation of innate and adaptive immunity. This latter point should be discussed and better clarified by the authors

We thank the reviewer for his/her comments.

Answer: This is a very relevant remark. We have therefore added the following text to the conclusion:

"Our findings are particularly important for the renal transplantation setting, as these procedures entail aortic clamping as well as stimulation of innate and adaptive immunity following transplantation. A pharmacological role of TEMPOL may therefore be even more limited in this context, and other therapeutics in addition to immunosuppressive drugs should be studied to protect both donor (in case of living donor kidney transplantation) and recipient."

Reviewer 3:

This study was conducted in order to evaluate the renoprotective effects of tempol against aortic occlusion-induced renal ischemia/reperfusion and the following renal damage.

The pre- and post-ischemic treatment with tempol at a cumulative dose of 350 mmol/kg produced a protective effect against renal damage, inflammation, and iNOS expression, although the beneficial effects on renal hemodynamics and oxidative stress.

The purpose of the study is clear, and methodology is also very well, findings are well discussed.

We thank to reviewer for his/her compliments and comments.

The rationale of the dose of tempol should be described in the method.

Answer: A rationale was provided in the Methods section.

In addition, the photograph of surgery is "Figure 2".

Answer: Thank you, we changed this accordingly.

2nd Editorial decision
Date: 06-September-2015

Ref.: Ms. No. JCTRes-D-15-00004R1
TEMPOL has limited protective effects on renal oxygenation and hemodynamics but reduces kidney damage and inflammation in a rat model of renal ischemia/reperfusion by aortic clamping
Journal of Clinical and Translational Research

Dear Dr. Ergin,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You have sufficiently addressed the concerns raised by one of our editors and the two reviewers, so your manuscript did not warrant further re-examination.

Thank you for submitting your work to JCTR and congratulations on the acceptance of your manuscript.

The proofs of your manuscript will be sent to you shortly.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research