**Author’s response to reviews**

**Title:** Pirfenidone prevents Acute Kidney Injury in the Rat

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**Author’s response to reviews:**

México City October 31th, 2018.

Dr. Hayley Henderson,

Editor-in-Chief

Dear Dr. Henderson:

We are re-submitting the following manuscript for consideration in the BMC Nephrology: Pirfenidone prevents Acute Kidney Injury in the Rat, BNEP-D-18-00428R1

We thank to the reviewers for their positive observations and comments that substantially improved the quality of our manuscript. All suggested changes were added and are highlighted in red font in the manuscript. In addition, a response letter point by point of each reviewer is included.

We appreciate the opportunity to publish our manuscript in BMC Nephrology.
Mark De Caestecker (Reviewer 1):

Major criticisms

1) Low rat numbers to assess functional recovery from IR-AKI:

There are no data available regarding the potential effects of Pirfenidone in preventing acute kidney injury due to I/R in rats. Considering our experience in the model of renal injury induced by I/R, we calculated the sample size by using comparing two means of expected values test. For this, creatinine clearance of control and I/R groups were used for a power level of 95% and alpha error of 0.05. The resulting sample size was 5 animals per group. So, we decide to study 6 animals in order to increase the power of the test and to follow the 3R’s principle in the use of experimental animals.

Minor criticisms

1) Marked tissue injury despite short clamp times (20 minutes) needs to be discussed in relation to other data from the same lab.

It is important to clarify that the susceptibility to the renal injury induced in the rat could be different among the breeding stocks. In our animal facility the breeding stock is changed every two years. Anyway, in our previous reports, the degree of renal injury induced by 20 min of bilateral renal ischemia was similar to this study. Renal injury induced by I/R was characterized by a significant reduction in renal blood flow and creatinine clearance, together with significative
proteinuria and extensive tubular injury. Part of this paragraph was added in the discussion section.

2) Background discussion about the central role of NaK ATPase on epithelial polarity is debatable and unnecessary for the background.

We deleted this information

3) Methods should specify the strain of rats used

We add the strain “Wistar rats” in methods

4) IR-AKI controls need to be vehicle treated and information about the volume and vehicle used for PF treatment needs to be described

An apologize for the missing information. The next paragraph was added in the methods section;

Pirfenidone was provided by Cell Pharma. Standard rat diet was powdered and the pirfenidone was homogeneously incorporated in it to reach the desired dose. The amount of food consumed daily by rats, according to age and weight, was previously determined by observation of a dedicated group of rats (approximately 20 g/day). The required dose of Pirfenidone was mixed with the amount of food that rats would consume in 24 h, in order to ensure appropriate drug exposure. The used dose of pirfenidone (700 mg/kg/day) was previously reported by Ji X, et al. and Takakuta K, et. al. {3162}{3165}. We acknowledge that half-life of Pirfenidone is quite short, and the desired dose was consumed by rats in the 24 h that preceded the I/R in a non-clearly determinable fashion. Nevertheless, since 80% of the dose of Pirfenidone is excreted by the urine, systemic exposure is substantially increased with severely impaired kidney function. Control and IR groups received the reconstructed pellets but without pirfenidone.

Reference: Renoprotective mechanisms of pirfenidone in hypertension-induced renal injury: through anti-fibrotic and anti-oxidative stress pathways Xu JI1, 2, Yukiko NAITO2, Huachun WENG2, Xiao MA2, Kosuke ENDO2, Naoko KITO2, Nariaki YANAGAWA2, Yang YU1, Jie LI1, and Naoharu IWA12 Biomedical Research 34 (6) 309-319, 2013

5) Rats are placed in metabolic cages 2 hours after surgery. I assume rats have been habituated to the metabolic cages beforehand. If so, please state this. If not, provide a rationale why this was not done
The following information was added to methods: All rats were allowed to acclimate in metabolic cages three days before the experiment.

6) Provide references for the use of urinary HSP72 as an AKI biomarker: it is not commonly used.

Thanks for your observation, we have added the references and the following sentence to clarify this issue.

The Hsp72 levels in urine were assessed by Western blot analysis as a marker of renal injury as we have previously reported.

7) Provide figure legends to illustrate graph bars

We have modified all the figure legends.

8) The statement that eNOS mRNA was reduced by T-Test but not ANOVA, is meaningless and should be removed. You can state that eNOS levels increased with PF treatment but this did not reach the level of statistical significance. That said, this could easily be a false negative result because of the small numbers of animals used

We removed this sentence and added “but not reach the level of statistical significance by ANOVA” instead in results section.

9) Provide scale bars in the microscopy images

We added the proper scale bars in the microscopy images

Rajesh Mohandas (Reviewer 2):

Major Concerns
Some key experimental details have not been included in the manuscript. What strain of rats were included? How old were they? These are important factors that determine the extent of renal injury beyond the ischemia time and I could not find where it was mentioned in the manuscript.

Sorry for the missing information. We added the strain “Wistar rats” and the age “10 to 12 weeks old” in methods section.

While the initial discussion focuses on decreased blood flow as the cause of ATN, the authors clamped the renal pedicle instead of just the renal artery. This would cause venous obstruction, induce renal venous congestion and increase the severity of renal injury. Was there a reason to favor clamping the pedicle versus the renal artery?

Clamping of renal pedicle was preferred to avoid unnecessary prolongation of surgery time in order to dissect the renal artery. Additionally, this model has been extensively studied in our laboratory giving us reproducible results with a lesser manipulation.

The authors report creatinine clearance. In rats there is significant secretion of creatinine. Moreover in acute kidney injury creatinine levels or excretion is not in steady state. Use of Dextran-FITC or inulin-FITC would have been ideal to determine GFR. The authors should report serum BUN and Creatinine in the animals and note the limitation in the discussion.

We appreciate your observation, serum BUN was measured in all blood samples. The results were added in Figure 1 and they were similar to the creatinine clearance.

"Pirfenidone was administered 24 hours before surgery at 700 mg/kg/day". How was this administered? In water? In food? How was the dose determined? Was a dose response assessed? The half-life of pirfenidone is short < 2 hours. How was it ensured that rats had pirfenidone when they underwent ischemia reperfusion.

We apologize for not clarify this important issue. We have added the following paragraph in the methods section:
Pirfenidone was provided by Cell Pharma. Standard rat diet was powdered and the pirfenidone was homogeneously incorporated in it to reach the desired dose. The amount of food consumed daily by rats, according to age and weight, was previously determined by observation of a dedicated group of rats (approximately 20 g/day). The required dose of Pirfenidone was mixed with the amount of food that rats would consume in 24 h, in order to ensure appropriate drug exposure. The used dose of pirfenidone (700 mg/kg/day) was previously reported by Ji X, et al. and Takakuta K, et. al. \cite{3162,3165}. We acknowledge that half-life of Pirfenidone is quite short, and the desired dose was consumed by rats in the 24 h that preceded the I/R in a non-clearly determinable fashion. Nevertheless, since 80% of the dose of Pirfenidone is excreted by the urine, systemic exposure is substantially increased with severely impaired kidney function. Control and IR groups received the reconstructed pellets but without pirfenidone.


What does the authors mean by the statement "mRNA levels were significant by t-test but not by ANOVA". An ANOVA and t-test should produce the same results if there are only two groups. If there are more than two groups and you do multiple t-tests then you increase the chances of Type-1 error. Such tests have to be adjusted for multiple post-hoc testing, which will compromise their power. So if the tests was not significant in ANOVA then the authors cannot claim any difference between groups.

We removed this statement and we added “but not reach the level of statistical significance by ANOVA” instead.

Figure-1 legend: The authors say both I/R group and the IR+PFN group as shown by black bars. I suspect the authors meant to say the former as black and the latter as grey. Details need to be specified in the figure legends as to how many mice were used and how the p-value was derived. Is this ANOVA? Multiple t-tests? According to the figure there is a significant increase in body weight with IR. Is that true? If so why would that be?
Sorry for the mistakes, the figure legends were corrected and now we added the number of animals and mention the statistics. On the weight, we added the initial weight of the animals, as shown, the rats subjected to IR were slightly heavier at the beginning of the experiment, therefore we consider that the fact of being slightly heavier at the end of the experiment is not relevant.

Figure-2C. There are no loading controls shown for the western blot. How were they normalized? AKI is associated with changes in proteinuria. So I would think a loading control/normalization is essential.

Accordingly with the reviewer, Hsp72 levels were normalized by the proteinuria and the Figure 2D was modified.

Figure-3. The authors show 3 figures representative of sham, IR and IR+PFN. However, figures A and C show cortex with plenty of glomeruli. The cortex will not contain the S2/S3 segments were damage is expected to be worse. Only Fig B with IR injury shows the medulla with casts containing tubules. We are not comparing apples to apples here.

We appreciate a lot your observation and we modified the representative microphotographs. In addition, the scale bar was added in each one.

Figure-4. The number of replicates is not mentioned. Also what statistical tests were done.

We made two replications and added the statistical test

Minor concerns

Page 3: Line 4: "AKI is caused by a reduction in blood flow". This statement is not entirely correct. Not all causes of AKI are characterized by a reduction in blood flow. There is ample evidence that sepsis is associated with increased renal blood flow. There is no decreased blood flow in AKI due to nephrotoxins.

We agree with the reviewer, the sentence was modified as: AKI is often caused by a reduction in renal........
There is no control group of Pirfenidone in sham operated rats. The effects of Pirfenidone could be independent of renal injury.

The rats were euthanized and studied 24 h after sham surgery or reperfusion. A sham operated group treated with PFN was not included because the 3R´s principle and because pirfenidone did not modify the basal renal function as has been previously reported.

I would examine the mRNA levels of SOD1 (in addition to Catalase and GPX) as well as hsp72.

We also examined SOD without statistically significant differences among the groups.

The authors speculate the protein in the urine is tubular in origin. This can be easily assessed by measuring urine micro-albumin creatinine ratio in addition to total.

We appreciate the observation of the reviewer, thus we measured urine micro-albumin in all the samples and the urinary micro-albumin/creatinine ratio was calculated. The results showed that micro-albumin/creatinine ratio increased significantly in the I/R group compared to control group. An effect that was partially prevented by pirfenidone. Accordingly, methods, Figure 2B, results, and discussion sections were modified.