Author's response to reviews

Title: Triggering of suicidal erythrocyte death by uremic toxin indoxyl sulfate

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Author's response to reviews: see over
Dear Ms Maria Merrie Jul Ladag and Miss Hayley Henderson

Thank you indeed for the evaluation of the paper:

**Triggering of suicidal erythrocyte death by uremic toxin indoxyl sulfate**
by
Mohamed Siyabeldin E. Ahmed, Majed Abed, Jakob Voelkl and Florian Lang

As detailed below, we have considered all comments raised by the referees. Thus, we do hope that you and the referees consider the paper now appropriate for publication in *BMC Nephrology*.

with kind regards

Florian Lang
Reviewer: 1

In this paper Ahmed et al analysed the effects of the uremic toxin indoxyl sulfate on the suicidal erythrocyte death (eryptosis). The authors show that a 48 hours exposure to indoxyl sulfate significantly increases cytosolic Ca2+ activity ([Ca2+]i), significantly increases ceramide formation, decreases cell volume and increases phosphatidylserine exposure at the erythrocyte surface. The effect of indoxyl sulfate on phosphatidylserine exposure was virtually abolished in the nominal absence of extracellular Ca2+. The authors conclude that indoxyl sulfate stimulates suicidal erythrocyte death or eryptosis thus contributing to the accelerated loss of circulating erythrocytes.

The present paper addresses an important topic. The experiments appear carefully done, the paper is well written and the conclusions of the paper are well supported by the results.

Minor Essential Revisions.

Several points need to be addressed prior to publication:

1. The concentrations of indoxyl sulfate required to stimulate annexin V binding are apparently lower than those required to increase Fluo3 fluorescence or forward scatter. The authors should comment on this seeming discrepancy.

   We now comment on the seeming discrepancy between the concentrations required to trigger annexin V binding and those required to increase Fluo3 fluorescence or forward scatter.

2. Inspection of Fig. 2 suggests that even at the highest concentrations applied, indoxylsulfate does not elicit hemolysis. The authors should comment on the lack of hemolysis.

   We now comment on the lack of hemolysis

3. The authors should comment on the putative quantitative contribution of eryptosis to anemia of patients with CKD.

   We now comment on the putative quantitative contribution of eryptosis to anemia of patients with CKD

4. In the discussion the authors focus on cytosolic Ca2+ as the trigger of eryptosis by indoxyl sulfate. This may be an oversimplification. The authors should discuss additional mechanisms possibly involved, such as ceramide or p38 MAP kinase.

   We now discuss additional mechanisms possibly involved, such as ceramide, caspases or kinases.

5. Several typos should be corrected, e.g.:...previously been shown... instead of...previously been shown... vascular wall at least in... instead of...vascular wall at least in... adherence to the vascular wall... instead of...adherence to the vascular wall...

   Typographical errors have been corrected as suggested

Reviewer: 2:

Triggering of suicidal erythrocyte death by uremic toxin indoxyl sulphate Dr Lang and his group have already published a very great deal on this subject. There are hundreds of uraemic
toxins and there is nothing to stop them examining ‘eryptosis’ by one toxin after another. Is this mechanism relevant to man; relevant to the clinic situation? I don’t know and I can find no relevant discussion of this in the paper.

We now amplified the introduction on the magnitude of eryptosis in patients on dialysis and its significance for the development of anemia in those patients.

This is a research paper and not a review article and it is absurd to have 94 references. Any resubmission should have no more than 30 references.

The number of references has been decreased substantially by replacing citations of original papers by reviews. A decrease to 30 is not possible without undue ignorance of published knowledge.

We need some practical information and discussion of how this process can be measured in man; or if it has been what has been shown. Ultimately (not necessarily in this paper) they have to show that some clinical measurement of eryptosis correlates with the blood concentration of indoxyl sulphate (or whatever toxin they are measuring). BMC is a nephrology journal and this paper needs to address the problem in a clinically relevant manner. At present this is an excellent biochemistry paper that needs to be published in a suitable biochemistry journal.

We now amplify in the introduction that eryptosis has been measured in patients and shown to be relevant for in vivo life span of erythrocytes.

Reviewer 3:

This study aims to explore whether indoxyl sulfate (IS), an organic anion toxin originated from the indole produced by intestinal bacteria as a metabolite of tryptophan, triggers the suicidal death of erythrocytes (eryptosis). Its rationale was that severe complications of end stage renal disease include anemia which may, at least partially, be due to enhanced eryptosis as an end result of the accumulation of IS in the blood of patients with chronic kidney disease. To this end, the effect of indoxyl sulfate on [Ca2+]i, cell volume, ceramide formation and phosphatidylserine abundance at the erythrocyte surface were determined in human leukocyte-depleted erythrocytes. According to the results shown, a 48 hours exposure to indoxyl sulfate significantly increased [Ca2+]i (# 300 µM), significantly decreased forward scatter (# 300 µM) and significantly increased annexin-V-binding (# 50 µM). IS (150 µM) induced annexin-V-binding was virtually abolished in the nominal absence of extracellular Ca2+. IS (150 µM) further enhanced ceramide abundance. Hence, the authors appropriately conclude stating that their study uncovers a novel effect of IS, i.e. the triggering of erythrocyte shrinkage and erythrocyte cell membrane scrambling, both hallmarks of suicidal erythrocyte death or eryptosis. The concentrations of indoxyl sulfate required for statistically significant stimulation of eryptosis were in the range of those encountered in uremic plasma. There are no specific concerns on originality, clarity of writing, clinical importance, study design, soundness of interpretation, and relevancy of discussion of the paper. Thus, I recommend this novel study for publication in BMC Nephrology.

Thank you for this judgement