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Ophthalmic acid as a read-out for hepatic glutathione metabolism in humans

Kim MC van Mierlo, Simon AWG Dello, Mechteld C de Jong, Hans MH van Eijk, Theo M de Kok, Jacob J Briedé, Frank G Schaap, Steven WM Olde Damink, Cornelius HC Dejong

Corresponding author
Steven WM Olde Damink
Department of Surgery, Maastricht University, NUTRIM School of Nutrition and Translational Research in Metabolism, 6229 ER Maastricht, the Netherlands

Handling editor
Michal Heger
Department of Experimental Surgery, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

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Dear authors,

Three experts in the field (liver toxicology and pharmacology) have now commented on your paper, yielding an accept, a major revision, and a reject. On the basis of their critical appraisal, I am advising that you revise your manuscript. Please pay particular attention to the points brought forward by reviewer #3, who raised doubt about the experimental design, back-translatability of the data to animal studies, and the premises the study was based on. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, 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. Also make sure that the changes in the manuscript are directly visible, e.g., by using the track changes function in Word.

Your revision is due by Dec 14, 2017.

To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item called 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: In brief, this is a small but important clinical study that sets out to test the human relevance of the finding of several important papers, intially Soga et al (2006) and then several others, using different methodologies and models, that ophthalmic acid (OA) inversely correlates with the level of hepatic glutathione (GSH) and that this represents a potential biomarker to read the status of a key abundant hepatic antioxidant.
This new and original study, as the above finding has not been explored clinically, explored the levels of OA and GSH during, pre- and post-liver resection surgery or pancreatic surgery, during co-administration of a therapeutic dose of acetaminophen (1g), a well-known depleter of hepatic glutathione at toxic doses, and which can deplete by approx 10%, the levels of hepatic GSH upon therapeutic administration.
This is important as we need to know if APAP administration may hinder liver function during this type of surgery and recovery, and because it may give insight into the relevance of the above-mentioned pre-clinical findings.
The key message of the paper is that in humans exposed to acetaminophen, contrary to the earlier studies, the fall in GSH occurs in a positively-correlated fashion with OA. The authors correctly state the lack of significant differences in their study, and that their finding is opposite to the earlier studeis, may be due to the therapeutic, rather than toxicological dose, that was administered in this human study. This is indeed quite possible, and further, they discuss their contradictory findings in relation to the methods used in the earlier studies.
In my opinion, I consider this to be satisfactory explanation, given the inherent limitations in human studies. I consider this to be an important addition to the field, it looks to have been well-designed and conducted, and I would not ask for revisions at this point. Therefore my recommendation is accept.

Reviewer #2: The authors measured plasma GSH and ophthalmic acid (OP) levels in patients that underwent hepatic resections (partial hepatectomy) or pylorus-preserving pancreaticoduodenectomy (PPPD) representing control surgery without loss of hepatic mass. All patients received doses of 1000 mg acetaminophen before and after surgery. The authors generally observed a correlation between GSH and OP but did not observe differences in plasma levels between hepatectomy surgery and PPPD. The authors concluded that in contrast to the expectation based on animal studies, the drop in plasma GSH after the first APAP dose did not result in an increase in OP. Generally, APAP did neither affect hepatic GSH nor OP levels.
1. This is an interesting study showing mainly the safety of APAP use in these surgery patients. As the authors concluded, the difference between the current clinical study and previous animal experiments is mainly the dose. The authors could also add the direct calculations: 1000 mg APAP in a patient with a 1500 g liver, which means approximately

0.67 mg APAP/g liver. If 5% of a therapeutic dose is converted to NAPQI, which reacts with GSH, about 0.22 µmol GSH/ g liver is being consumed. If the measured hepatic GSH levels of 1.5 µmol GSH/ g liver are being considered, this means that about 15% of the hepatic GSH levels are being used. This is by far less than the >90% of hepatic GSH loss after an overdose in mice. This rough estimate explains why the current results are not comparable to the animal studies.
2. The authors speculate that the results may be different after an APAP overdose in patients. This has actually been done: Kaur et al., Detection of Ophthalmic Acid in Serum from Acetaminophen-Induced Acute Liver Failure Patients Is More Frequent in Non-Survivors. PLoS One. 2015 Sep 25;10(9):e0139299. This study should be cited and the results discussed in the paper.
3. Line 350-351: The authors state that "APAP caused hepatocellular damage even in healthy patients (ref.30-32)". However, this statement is too general and inaccurate. First, 2 of the cited papers are just comments not clinical studies. Second, ref. 30 reported elevated ALT levels in some patients receiving therapeutic doses of APAP, but none of the patients suffered from severe liver injury or even liver failure. This statement needs to be more accurately rephrased.

Reviewer #3: The current manuscript evaluates ophthalmic acid as a read-out for hepatic glutathione metabolism in humans in patients undergoing partial hepatectomy in comparison to those undergoing pancreatic surgery as controls. While the main rationale for the study has been purported to be the increased susceptibility of hepatectomy patients to acetaminophen-induced liver injury, this is questionable, indicating a serious flaw in experimental design.

Major comment
1. The authors main rationale for carrying out this study is based on their statement that "In the presence of a diminished liver volume and additional surgical stress during partial hepatectomy (PH), the administration of a normal dose of APAP has been suggested to lead to a faster depletion of hepatic GSH stores (17)". However, the reference (17) they quote for this statement is a review on ischemia-reperfusion injury, which has no mention of acetaminophen- and hence the basis for this assumption is unclear.

2. This is all the more confusing when, in fact, it has already been shown that- while low residual liver volume results in altered acetaminophen metabolism, no evidence of glutathione deficiency was observed, with the conclusion that therapeutic acetaminophen is safe after major liver resection provided liver function is adequate (1). Hence, the novelty and rationale for carrying out these experiments in the first place is not very clear.

3. Another main issue regarding experimental design is the fact that animal studies examining ophthalmic acid as a measure of glutathione were doing it in the context of an acetaminophen overdose, when excessive formation of the reactive metabolite depletes liver glutathione stores. In the current study, where therapeutic acetaminophen doses were obviously used, reactive metabolite formation would be expected to be negligible, without any effect on glutathione stores.

References
1. Hughes MJ, Harrison EM, Jin Y, Homer N, Wigmore SJ. Acetaminophen metabolism after liver resection: A prospective case-control study. Dig Liver Dis. 2015 Dec;47(12):1039-46.

Authors' response

Reply to the reviewers.

We are indebted to all reviewers for their valuable comments that helped to improve the quality of the manuscript.

Reviewer #1

1.1 In brief, this is a small but important clinical study that sets out to test the human relevance of the finding of several important papers, initially Soga et al (2006) and then several others, using different methodologies and models, that ophthalmic acid (OA) inversely correlates with the level of hepatic glutathione (GSH) and that this represents a potential biomarker to read the status of a key abundant hepatic antioxidant. This new and original study, as the above finding has not been explored clinically, explored the levels of OA and GSH during, pre- and post-liver resection surgery or pancreatic surgery, during co-administration of a therapeutic dose of acetaminophen (1g), a well-known depleter of hepatic glutathione at toxic doses, and which can deplete by approx 10%, the levels of hepatic GSH upon therapeutic administration. This is important as we need to know if APAP administration may hinder liver function during this type of surgery and recovery, and because it may give insight into the relevance of the abovementioned pre-clinical findings. The key message of the paper is that in humans exposed to acetaminophen, contrary to the earlier studies, the fall in GSH occurs in a positively-correlated fashion with OA. The authors correctly state the lack of significant differences in their study, and that their finding is opposite to the earlier studies, may be due to the therapeutic, rather than toxicological dose, that was administered in this human study. This is indeed quite possible, and further, they discuss their contradictory findings in relation to the methods used in the earlier studies. In my opinion, I consider this to be satisfactory explanation, given the inherent limitations in human studies. I consider this to be an important addition to the field, it looks to have been well-designed and conducted, and I would not ask for revisions at this point.
Therefore my recommendation is accept.
Thank you for your critical review and appreciating the value of the study, and the limitations inherent to human studies.

Reviewer #2

2.1 The authors measured plasma GSH and ophthalmic acid (OP) levels in patients that underwent hepatic resections (partial hepatectomy) or pylorus-preserving pancreaticoduodenectomy (PPPD) representing control surgery without loss of hepatic mass. All patients received doses of 1000 mg acetaminophen before and after surgery. The authors generally observed a correlation between GSH and OP but did not observe differences in plasma levels between hepatectomy surgery and PPPD. The authors concluded that in contrast to the expectation based on animal studies, the drop in plasma GSH after the first APAP dose did not result in an increase in OP. Generally, APAP did neither affect hepatic GSH nor OP levels. This is an interesting study showing mainly the safety of APAP use in these surgery patients. As the authors concluded, the difference between the current clinical study and previous animal experiments is mainly the dose. The authors could also add the direct calculations:

1000 mg APAP in a patient with a 1500 g liver, which means approximately 0.67 mg APAP/g liver. If 5% of a therapeutic dose is converted to NAPQI, which reacts with GSH, about 0.22 µmol GSH/ g liver is being consumed. If the measured hepatic GSH levels of 1.5 µmol GSH/ g liver are being considered, this means that about 15% of the hepatic GSH levels are being used. This is by far less than the 70->90% of hepatic GSH loss after an overdose in mice. This rough estimate explains why the current results are not comparable to the animal studies.

Thank you for your critical review and helpful suggestions. We added the calculation in the discussion section of the manuscript to clarify the results.

Text added on page 18.

> *"Assuming an average adult liver weight of 1500 g, and conversion of 5% of ingested APAP (i.e. 50 mg = 0.33 mmoL) into GSH-consuming NAPQI, we estimated that the initial APAP dose resulted in consumption of 0.22 µmol GSH/g liver. Considering hepatic GSH levels of 1.5 µmol GSH/g liver (Figure 3A), about 15% of hepatic GSH content would be consumed. This is far less than the hepatic GSH depletion (>90%) after an APAP overdose in mice.[12]"*

2.2 The authors speculate that the [serum] results may be different after an APAP overdose in patients. This has actually been done: Kaur et al., Detection of Ophthalmic Acid in Serum from Acetaminophen-Induced Acute Liver Failure Patients Is More Frequent in NonSurvivors. PLoS One. 2015 Sep 25;10(9):e0139299. This study should be cited and the results discussed in the paper.

We were aware of this study, wherein serum ophthalmic acid levels were studied in survivors and non-survivors of APAP-induced acute liver failure. Non-survivors were more likely to have detectable ophthalmic acid levels than survivors at day 2 and day 4 after hospitalization. Due to the differences in administration (multiple therapeutic doses vs. bolus overdose) and interval between APAP ingestion and serum analysis in our study and that of Kaur et al., we decided (then and now) against including the study. This was corroborated by the fact that detectability was not an issue in our study, with OA detected in all serum samples (range: 30-70 µmol/L). In contrast, OA could be detected in 11-31% of the sera at day 2 and day 4 after hospitalization (i.e. at least 2 days after APAP overingestion), with levels in "OA positive samples" in the submicromolar range (0.36-0.48 µmol/L).

2.3 Line 350-351: The authors state that "APAP caused hepatocellular damage even in healthy patients (ref.30-32)". However, this statement is too general and inaccurate. First, 2 of the cited papers are just comments not clinical studies. Second, ref. 30 reported elevated ALT levels in some patients receiving therapeutic doses of APAP, but none of the patients suffered from severe liver injury or even liver failure. This statement needs to be more accurately rephrased.

We agree with the reviewer that this statement required nuancing, and rephrased the sentence as follows (page 20):

> *"Even in healthy individuals, peak ALT elevations up to 8-fold were reported in 27% of participants receiving a therapeutic dose of APAP.[30]"*

Reviewer #3

3.1 The current manuscript evaluates ophthalmic acid as a read-out for hepatic glutathione metabolism in humans in patients undergoing partial hepatectomy in comparison to those undergoing pancreatic surgery as controls. While the main rationale for the study has been purported to be the increased susceptibility of hepatectomy patients to acetaminopheninduced liver injury, this is questionable, indicating a serious flaw in experimental design. The authors main rationale for carrying out this study is based on their statement that "In the presence of a diminished liver volume and additional surgical stress during partial hepatectomy (PH), the administration of a normal dose of APAP has been suggested to lead to a faster depletion of hepatic GSH stores (17)". However, the reference (17) they quote for this statement is a review on ischemia-reperfusion injury, which has no mention of acetaminophen- and hence the basis for this assumption is unclear.

Thank you for your critical evaluation of our manuscript. The study was designed to evaluate whether OP levels can be used as a read-out for hepatic GSH homeostasis in patients undergoing liver resection. Although the reviewer may disagree with the rationale for our hypothesis that patients undergoing liver resection are more susceptible to APAP-induced liver injury, we disagree that there is a serious flaw in experimental design and are confident that the set-up of the study was -and is- appropriate to prove or refute our hypothesis. Insights from the study by Hughes et al. (published in 2015, reviewer's reference 1), indicating that low residual liver volume does not result in glutathione deficiency, were not available at the time that we conducted our study (in 2012).

3.2 This is all the more confusing when, in fact, it has already been shown that- while low residual liver volume results in altered acetaminophen metabolism, no evidence of glutathione deficiency was observed, with the conclusion that therapeutic acetaminophen is safe after major liver resection provided liver function is adequate (1). Hence, the novelty and rationale for carrying out these experiments in the first place is not very clear.

As mentioned above, insights from the study by Hughes et al. (published in 2015, reviewer's reference 1) were not available at the time that we conducted our study (in 2012). Independent replication of studies is especially relevant in case of observations with potential clinical implication. Not only does our study confirm that there is no significant decrease of serum glutathione after liver resection, added novelty of our study is that we show lack of acute glutathione depletion in the liver and unaltered levels of ophthalmic acid in liver or the circulation. Hence, our findings support the notion of Hughes et al. that use of APAP is safe in patients undergoing major liver resection, provided that liver function is adequate.

Text added on page 19.

> *Hence, our findings support the notion of Hughes et al. who concluded that use of APAP is safe in patients undergoing major liver resection, provided that liver function is adequate.*[28]

3.3 Another main issue regarding experimental design is the fact that animal studies examining ophthalmic acid as a measure of glutathione were doing it in the context of

an acetaminophen overdose, when excessive formation of the reactive metabolite depletes liver glutathione stores. In the current study, where therapeutic acetaminophen doses were obviously used, reactive metabolite formation would be expected to be negligible, without any effect on glutathione stores.
We agree with the reviewer that the relatively low (safe) dose of acetaminophen may be the most important reason for a lack of heaptic glutathione depletion and increase in ophthalmic acid increase. In the discussion this possibility was further expanded by estimating the APAPinduced decrease in hepatic GSH content, as detailed in our reply to reviewer #2.

By administering this dose concurrently with hepatic surgery and the associated stress that this causes, we anticipated to deplete the glutathione storage just enough to prove the principle of a counter rise in ophthalmic acid. However, this was unsuccessful. Obviously, increasing APAP dose was not justifiable.

Text added on page 18.

> *"Assuming an average adult liver weight of 1500 g, and conversion of 5% of ingested APAP (i.e. 50 mg = 0.33 mmoL) into GSH-consuming NAPQI, we estimated that the initial APAP dose resulted in consumption of 0.22 µmol GSH/g liver. Considering hepatic GSH levels of 1.5 µmol GSH/g liver (Figure 3A), about 15% of hepatic GSH content would be consumed. This is far less than the hepatic GSH depletion (>90%) after an APAP overdose in mice.[12]"*

Reference

1. Hughes MJ, Harrison EM, Jin Y, Homer N, Wigmore SJ. Acetaminophen metabolism after liver resection: A prospective case-control study. Dig Liver Dis. 2015 Dec;47(12):1039-46.

2nd Editorial decision

Date: 23-Jan-2018

Ref.: Ms. No. JCTRes-D-17-00015R1
Ophthalmic acid as a read-out for hepatic glutathione metabolism in humans
Journal of Clinical and Translational Research

Dear authors,

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

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly review for any errors.

Thank you for submitting your work to JCTR.

Kindest regards,

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

Comments from the editors and reviewers:

Reviewer #2: The authors satisfactorily addressed the reviewers' comments.