

Connexin-based signaling and drug-induced hepatotoxicity

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Journal of Clinical and Translational Research

Dear Dr. Vinken,

Reviewers have submitted their critical appraisal of your paper. The reviewers' comments are appended below. Based on their comments and evaluation by the editorial board, your work was FOUND SUITABLE FOR PUBLICATION AFTER MINOR REVISION. One issue, besides the comments of the reviewers, is the fact that Figure 1 appears to be identical to a previously published figure (Maes et al., *Toxicol In Vitro*. 2015 Dec 25;30:569-77). The authors need to get permission to republish the figure and properly cite the original source.

If you decide to revise the work, please itemize the reviewers' comments and provide a point-by-point response to every comment. An exemplary rebuttal letter can be found on at <http://www.jctres.com/en/author-guidelines/> under "Manuscript preparation." Also, please use the track changes function in the original document so that the reviewers can easily verify your responses.

Your revision is due by Jan 14, 2017.

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

Hartmut Jaeschke, PhD

Associate Editor

Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: JCTRes-D-16-00027

Connexin-based signaling and drug-induced hepatotoxicity

In in this review, the authors discuss what is currently known regarding the roles for connexins in drug-induced liver injury (DILI). A recent review of the available literature demonstrates that this is a relatively new area of research in which reviews are rare. Therefore, what is known regarding connexins in DILI is worthy of review. The comments below will help the authors revise this manuscript. In general, the comments are in terms of the order in which certain material is presented, content and finally, clarity.

Major comments

1. Introduction: Is there anything known regarding the roles for the different portions of the connexin molecule? For example, are any particular domains required for connexin interaction with other connexins to form hemichannels or gap junctions. A bit more information on connexin biology in this regard would be nice.

2.2 Regulatory properties. The authors should introduce this section by stating that both transcriptional and post translational mechanisms regulate connexins, for this is what is discussed in the following two paragraphs. Then, they should discuss transcriptional regulation before post translational mechanisms. Finally, the authors discuss the role of phosphorylation in the regulation of connexin function, but state that s-nitrosylation and sumoylation are also involved. The authors should include some information on the role that s-nitrosylation and sumoylation play in connexin regulation. Are these the only known ways connexins are regulated? If this is a gap in current knowledge, this might be a good thing to mention in the conclusions and perspectives section.

2.2 Regulatory properties. The authors make it a point to say that, "With the exception of Cx26, all known connexins are phosphoproteins..." (p. 4, lines 53+) but the on page 5 say that Cx43 is mostly non-phosphorylated in liver. Please make is clearer what the distinction between Cx26 and Cx43 is regarding their phosphorylation status.

2.3 Functional properties. This section requires reorganization. The authors should start the section using the second paragraph ("The establishment of GJIC..."). This should be followed by the section on liver regeneration and finally the paragraph on cell death. In each case, the paragraphs should end with a good summary statement.

2.3 Functional properties. What is the presumed/known role(s) for Cx32 hemichannels in cell death? This information should be included in the paragraph about connexins and cell death.

3.1 Acetaminophen. Assuming this information is known, it would be helpful to comment on the induction patterns of the various liver (or just hepatocyte) connexins in a time course after APAP exposure before jumping into the contrasting results with various genetic and pharmacologic approaches to tease out a role for the various proteins in DILI.

3.1 Acetaminophen. This paragraph shows both positive and negative aspects of connexins in APAP-induced liver injury. It would be helpful to tell the readers this point in the second sentence (before "Upon administration of APAP to...", page 7 line 21, so they are prepared for what follows.

3.1 Acetaminophen. Page 7, line 38. "This points to a role for gap junction-based signaling in hepatocyte death by distribution of either death signaling molecules or survival messengers between hepatocytes." How would 'survival messengers' facilitate hepatocyte death?

3.1 Acetaminophen. I think this section should end with a few sentences which try to make sense of the opposing roles for connexins in hepatocyte cell death. The conclusions/perspectives section has some of this information. It should be moved from the conclusions/perspectives section to this

section.

3.2 Hypolipidemic drugs. The first couple of sentences are awkward and need to be rewritten. Perhaps something like this: "PPARalpha agonists such as Clofibrate, nafenopin, and wy-14,643 are lipid-lowering agents which drive the expression of genes in fatty acid transport, binding and beta oxidation.

3.2 Hypolipidemic drugs. A summary statement is required. Are you trying to say that loss of gap junctions promotes HCC? How does this compare/contrast to the role connexins play in liver regeneration?

3.3 Phenobarbital. This section needs revision of grammar/usage. For example the first sentences could be more clear if written "Phenobarbital or phenobarbitone (PB) is an anti-epileptic drug that has sedative and hypnotic properties. It is frequently used as a model tumor promoter in rodent liver and alters expression of a broad set of genes, of which, those related to CYP xenobiotic biotransformation have gained most attention." Line 43 page 9: Perhaps the sentence immediately after reference 111 could say something like: "Interestingly (or however), connexins are required for PB-mediated tumor promotion" and then dive into the data which support that statement.

3.4 Methapyrilene. Is there additional information on connexins and liver injury caused by this drug? Only one sentence is provided which discusses methapyrilene in his section. Can this be expanded?

4. Conclusions and perspectives. As noted above, some of the material presented in this section might be more useful if presented in the other sections of the review. Perhaps this section could be used to summarize current knowledge gaps and areas for further research. Roles of panexins in DILI is a perfect thing to discuss in this regard. The paragraph beginning on page 11 line 46 is also perfect for this section.

Minor comments

The manuscript would benefit from some revision of grammar and usage.

It would be great if the authors could avoid using the phrase "connexin species". "Connexins" is sufficient and will not be confused with species when used to describe different animals used to study connexins.

Published literature should always be discussed in present tense (page 10 line 14).

Methapyrilene is an antihistamine (as opposed to an antihistaminic drug as found page 10 line, 29)

Page 11 line 26. "building stones" might be better if written "building blocks".

Reviewer #2: Thank you very much for submitting interesting paper for the gap junctions in liver from different aspects, such as drugs induced hepatotoxicity, hepatocellular carcinoma. However, I have a few concerns in your review.

1) Table1

In the table1, hepatocytes are not listed in the column of Cx43. Do you have any intention?

In this article (Page 5, Line 7-) , " in liver, Cx43 is mostly presented as the nonphosphorylated variant (52,52)" . In these papers (52,53), they demonstrated that Cx43 was expressed in rat hepatocytes. In addition, it was shown that Cx43 expression in hepatocytes using hepatocyte-specific marker (HepaCAM) by flow cytometer (99). However, I recommended whether other groups also

showed the expression of Cx43 in hepatocytes or not.

2) P7. Line 31-

"In addition, an in vitro study showed protection against synchronized necrotic cell death of attached hepatocytes originating from Cx32-/- mice compared to WT hepatocytes treated with APAP (88)"
I went through this article and it was shown that there was no difference, in terms of cell viability, between hepatocytes from WT mice and hepatocytes from Cx32-/- mice with Cx26 siRNA after APAP exposure. The results indicated that the synchronization of APAP induced necrotic cell death was not occurred with dysfunctional gap junctions.

3) P7. Line 58-P8. Line4

"However, our group recently found that Cx32-/- mice from less protein adducts, which could indicate a lower metabolic activity upon genetic ablation of Cx32 (94)"

It will be better to mention that this article showed less protein adducts at 6h after APAP administration in Cx32 -/- mice, however, there was no difference at 1h after APAP. It indicated that the metabolism of APAP was not disturbed or induced in Cx32 -/- KO mice.

*****Authors' rebuttal*****

The authors greatly acknowledge both reviewers for the evaluation of the paper and for providing useful comments. The following modifications have been made:

Reviewer #1

1. Introduction: Is there anything known regarding the roles for the different portions of the connexin molecule? For example, are any particular domains required for connexin interaction with other connexins to form hemichannels or gap junctions. A bit more information on connexin biology in this regard would be nice.

The authors agree with the reviewer. Therefore, the following parts have now been included in the introduction: "Following synthesis, 6 connexins form a hemichannel at the plasma membrane surface, which then docks with another hemichannel from a neighboring cell to generate a gap junction [18-20] (Figure 1). This occurs at the extracellular domains, where conserved cysteine residues create disulfide bonds [21]." Furthermore, the following has been added: "Despite some structural variation between connexins, the first extracellular loop, the first transmembrane domain, the cytosolic aminotail and/or the cytosolic loop are considered to contribute to hemichannel pore opening [27]."

2. Regulatory properties. The authors should introduce this section by stating that both transcriptional and post translational mechanisms regulate connexins, for this is what is discussed in the following two paragraphs. Then, they should discuss transcriptional regulation before post translational mechanisms. Finally, the authors discuss the role of phosphorylation in the regulation of connexin function, but state that s-nitrosylation and sumoylation are also involved. The authors should include some information on the role that s-nitrosylation and sumoylation play in connexin regulation. Are these the only known ways connexins are regulated? If this is a gap in current knowledge, this might be a good thing to mention in the conclusions and perspectives section.

The authors agree with the reviewer and have now introduced the following: "Connexin signaling can be regulated by a plethora of mechanisms at the transcriptional, posttranscriptional, translational and posttranslational level. As such, 2 major kinetic sources

of regulation have been described, namely short-term control (*i.e.* millisecond to minute range) and long-term control (*i.e.* hour range). They cooperate to fine-tune the degree of intercellular communication by controlling the number of channels, their functional state and their unitary permeability [44, 45].” Furthermore, transcriptional regulation is now discussed before the posttranslational mechanisms. In addition, the authors have included information regarding the involvement of S-nitrosylation and sumoylation in connexin signaling: “S-nitrosylation occurs at intracellular cysteine residues and is mediated by nitric oxide, which might be the underlying mechanism of increased hemichannel opening induced by metabolic inhibition and inflammatory conditions [60, 61]. Irreversible conjugation of small ubiquitin-like modifiers to lysine residues, so-called sumoylation, regulates Cx43 levels and the number of Cx43-based gap junctions at the plasma membrane [62]. Phosphorylation encompasses the addition of phosphate groups to polar amino acid side chains, among which serine, threonine and tyrosine residues. This posttranslational modification almost uniquely takes place at the cytoplasmic carboxyterminal tail.”

3. Regulatory properties. The authors make it a point to say that, “With the exception of Cx26, all known connexins are phosphoproteins...” (p. 4, lines 53+) but the on page 5 say that Cx43 is mostly non-phosphorylated in liver. Please make is clearer what the distinction between Cx26 and Cx43 is regarding their phosphorylation status.

The authors only partially agree with the reviewer. As stated, with the exception of Cx26, all known connexins can be phosphorylated by a broad panel of kinases. This does not mean that all connexins are phosphorylated in resting state *per se*. In this respect, Cx43 is mostly presented in the nonphosphorylated isoform in quiescent state in liver. However, in certain conditions, such as after acetaminophen overdosing, the Cx43 phosphorylation status is altered.

4. Functional properties. This section requires reorganization. The authors should start the section using the second paragraph (“The establishment of GJIC...”). This should be followed by the section on liver regeneration and finally the paragraph on cell death. In each case, the paragraphs should end with a good summary statement.

The authors agree with the reviewer and have reorganized the section “2.3 Functional properties” as suggested.

5. Functional properties. What is the presumed/known role(s) for Cx32 hemichannels in cell death? This information should be included in the paragraph about connexins and cell death.

The authors have included the requested information, namely: “Concomitantly, Cx32 is *de novo* synthesized and gathers in a hemichannel configuration. This becomes particularly evident towards the final stages of the cell death process, where Cx32 hemichannels facilitate the apoptosis-to-necrosis transition [92, 96].”

6. Acetaminophen. Assuming this information is known, it would be helpful to comment on the induction patterns of the various liver (or just hepatocyte) connexins in a time course after APAP exposure before jumping into the contrasting results with various genetic and pharmacologic approaches to tease out a role for the various proteins in DILI.

The authors agree with the reviewer and have included the following: “After APAP intoxication in rodents, a switch in mRNA and protein production from Cx32 and Cx26 to Cx43 is observed [93, 99]. The upregulation of Cx43 quantities is due to recruitment of Cx43-expressing inflammatory cells, but also originates from *de novo* production of hepatocytes [99].”

7. Acetaminophen. This paragraph shows both positive and negative aspects of connexins in APAP-induced liver injury. It would be helpful to tell the readers this point in the second sentence (before "Upon administration of APAP to...", page 7 line 21, so they are prepared for what follows.

The authors agree with the reviewer. Therefore, the authors introduced this issue before describing this in more detail: "A limited number of reports have described a role for Cx32-based gap junction in APAP-triggered hepatotoxicity using genetically modified animals, albeit with contradicting outcomes [93, 100-102]."

8. Acetaminophen. Page 7, line 38. "This points to a role for gap junction-based signaling in hepatocyte death by distribution of either death signaling molecules or survival messengers between hepatocytes." How would 'survival messengers' facilitate hepatocyte death?

The authors understand the reviewer's concern. The study of Saito and colleagues indeed showed that gap junctions could either distribute death signaling molecules or survival messengers between hepatocytes. The latter was evidenced by showing that APAP-sensitive male hepatocytes were protected by attachment to APAP-insensitive female hepatocytes, with this protection being dependent on gap junctions. The latter feature has been added to the description of the paper of Saito and colleagues as follows: "In addition, an *in vitro* study showed protection against synchronized necrotic cell death of attached hepatocytes originating from Cx32^{-/-} mice compared to WT hepatocytes treated with APAP. This synchronization of cell death was mediated by gap junctions formed of Cx26 and Cx32. Furthermore, APAP-sensitive male hepatocytes were protected by attachment to APAP-insensitive female hepatocytes, with this protection being dependent on gap junctions. This points to a role for gap junction-based signaling in hepatocyte death by distribution of either death signaling molecules or survival messengers between hepatocytes [94]."

9. Acetaminophen. I think this section should end with a few sentences which try to make sense of the opposing roles for connexins in hepatocyte cell death. The conclusions/perspectives section has some of this information. It should be moved from the conclusions/perspectives section to this section.

The authors partially agree with the reviewer. The authors have added a conclusive sentence to this paragraph, namely: "In essence, *de novo* produced Cx43 after APAP-induced liver toxicity seems to have a protective role, while contradictory results were found with respect to the role of Cx32-based signaling." However, the authors prefer to keep the discussion about opposite actions of gap junctions/hemichannels in the conclusions/perspectives section, as this is one of the main messages of the paper. It might become lost if put in a smaller paragraph.

10. Hypolipidemic drugs. The first couple of sentences are awkward and need to be rewritten. Perhaps something like this: "PPAR α agonists such as Clofibrate, nafenopin, and wy-14,643 are lipid-lowering agents which drive the expression of genes in fatty acid transport, binding and beta oxidation.

The authors agree with the reviewer and have adapted the first sentences as suggested: "Peroxisome proliferator-activated receptor α agonists, such as clofibrate [107], nafenopin [108] and Wy-14,643 [109] are lipid-lowering agents, which drive the expression of genes involved in fatty acid transport, binding and β -oxidation in favor of proliferative activity."

11. Hypolipidemic drugs. A summary statement is required. Are you trying to say that loss of gap junctions promotes HCC? How does this compare/contrast to the role connexins play in liver regeneration?

The authors agree with the reviewer and have introduced the following summary statement,

together with an additional possible mechanistic interpretation of the effect of peroxisome proliferators: "Overall, peroxisome proliferators seem to perturb GJIC and alter hepatic connexin expression. Stimulation of hepatocyte proliferation by these agents has also been shown to be mediated, at least in part, by tumor necrosis factor α (TNF α) [116, 117]. Therefore, a conceivable explanation is that the downregulation of the connexin signaling is driven by TNF α released in response to peroxisome proliferators [114, 118, 119]. Indeed, TNF α treatment has been shown to modulate GJIC and to downregulate connexin gene expression [120]. Hence, GJIC inhibition by TNF α and subsequent promotion of hepatocyte proliferation might be a possible mechanistic interpretation of the effects of peroxisome proliferators on liver." In fact, this mechanistic interpretation might be in line with the role that connexins play in liver regeneration. Following hepatotoxic CCl₄ treatment or partial hepatectomy, a decrease in connexin content has also been observed in regenerating liver. Possibly, this downregulation is partly regulated by increased TNF α amounts in the regenerating liver.

12. Phenobarbital. This section needs revision of grammar/usage. For example the first sentences could be more clear if written "Phenobarbital or phenobarbitone (PB) is an anti-epileptic drug that has sedative and hypnotic properties. It is frequently used as a model tumor promoter in rodent liver and alters expression of a broad set of genes, of which, those related to CYP xenobiotic biotransformation have gained most attention." Line 43 page 9: Perhaps the sentence immediately after reference 111 could say something like: "Interestingly (or however), connexins are required for PB-mediated tumor promotion" and then dive into the data which support that statement.

The authors thank the reviewer for the grammatical correction. The first sentences have been adapted as suggested: "Phenobarbital or phenobarbitone (PB) is an anti-epileptic drug that has sedative and hypnotic properties. It is frequently used as a model tumor promoter in rodent liver and alters the expression of a broad set of genes [116, 117], of which, those related to cytochrome P450-dependent xenobiotic biotransformation have gained most attention [118]." The following has also been adapted: "Interestingly, connexins are required for PB-mediated tumor promotion."

13. Methapyrilene. Is there additional information on connexins and liver injury caused by this drug? Only one sentence is provided which discusses methapyrilene in his section. Can this be expanded?

The authors understand the reviewer's concern. Unfortunately, not much additional information can be given as no other studies have investigated the effect of methapyrilene on connexin signaling. However, additional details of this particular study have been added to this paragraph, namely: "With respect to intercellular communication mediated by gap junctions, it has been found that the number and size of Cx32-containing gap junction plaques in liver are negatively affected upon treatment of male rats with a carcinogenic dose of methapyrilene. However, this dose also increased the occurrence of apoptosis, which may also contribute to the negative affect of methapyrilene on liver gap junctions [142]."

14. Conclusions and perspectives. As noted above, some of the material presented in this section might be more useful if presented in the other sections of the review. Perhaps this section could be used to summarize current knowledge gaps and areas for further research. Roles of panexins in DILI is a perfect thing to discuss in this regard. The paragraph beginning on page 11 line 46 is also perfect for this section.

The authors do not fully agree with the reviewer. As stated in response to remark 9, the authors would prefer to keep some of the information in the conclusions/perspectives section

rather than shifting this to other sections. As the conclusion section is one of the most essential parts in the paper, the authors think it is important to preserve the main message in this part, in particular regarding the differential role of gap junctions compared to hemichannels.

15. The manuscript would benefit from some revision of grammar and usage.

The authors have now revised the grammar throughout the entire manuscript and made necessary changes.

16. It would be great if the authors could avoid using the phrase "connexin species". "Connexins" is sufficient and will not be confused with species when used to describe different animals used to study connexins.

The authors agree with the reviewer and have now replaced "connexin species" by "connexins".

17. Published literature should always be discussed in present tense (page 10 line 14).

The authors agree with the reviewer and have changed this to the present tense.

18. Methapyrilene is an antihistamine (as opposed to an antihistaminic drug as found page 10 line, 29).

The authors agree with the reviewer and have adapted this in the manuscript.

19. Page 11 line 26. "building stones" might be better if written "building blocks".

The authors agree with the reviewer and have adapted this in the manuscript.

Reviewer #2

1. In the table1, hepatocytes are not listed in the column of Cx43. Do you have any intention? In this article (Page 5, Line 7-) , " in liver, Cx43 is mostly presented as the nonphosphorylated variant (52,52)" . In these papers (52,53), they demonstrated that Cx43 was expressed in rat hepatocytes. In addition, it was shown that Cx43 expression in hepatocytes using hepatocyte-specific marker (HepaCAM) by flow cytometer (99). However, I recommended whether other groups also showed the expression of Cx43 in hepatocytes or not.

The authors have not listed hepatocytes as being Cx43 positive. Indeed, hepatocytes do not naturally express Cx43. However, in stress conditions, such as upon *in vitro* cultivation and in chemical-induced hepatotoxicity, Cx43 is *de novo* expressed by hepatocytes. Cx43 presence in liver, mainly in the nonphosphorylated form, fully originates from nonparenchymal cells, and thus not from hepatocytes.

2. P7. Line 31

"In addition, an *in vitro* study showed protection against synchronized necrotic cell death of attached hepatocytes originating from Cx32-/- mice compared to WT hepatocytes treated with APAP (88)". I went through this article and it was shown that there was no difference, in terms of cell viability, between hepatocytes from WT mice and hepatocytes from Cx32-/- mice with Cx26 siRNA after APAP exposure. The results indicated that the synchronization of APAP induced necrotic cell death was not occurred with dysfunctional gap junctions.

The authors appreciate the reviewer's comment. However, it is not clear to the authors what kind of adaptation is specifically suggested by the reviewer.

3. P7. Line 58-P8. Line4

"However, our group recently found that Cx32^{-/-} mice from less protein adducts, which could indicate a lower metabolic activity upon genetic ablation of Cx32 (94)". It will be better to mention that this article showed less protein adducts at 6h after APAP administration in Cx32^{-/-} mice, however, there was no difference at 1h after APAP. It indicated that the metabolism of APAP was not disturbed or induced in Cx32^{-/-} KO mice.

The authors understand the reviewer's concern. Therefore, the authors have included the details regarding the time point in these experiments, whereby differences were found in protein adducts.

2nd Editorial decision

Date: 28 Sep, 2016

Ref.: Ms. No. JCTRes-D-16-00027R1

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Journal of Clinical and Translational Research

Dear Dr. Vinken,

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

Comments from the editor and reviewers can be found below.

Thank you for submitting your work to JCTR.

Kindest regards,

Hartmut Jaeschke, PhD

Associate Editor

Journal of Clinical and Translational Research

Comments from the editors and reviewers:

The authors have addressed the comments of the reviewers and improved the manuscript.
