CORRESPONDENCE

REVISED Matching target dose to target organ [version 2; referees: 2 approved]

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Abstract

In vitro assays have become a mainstay of modern approaches to toxicology with the promise of replacing or reducing the number of *in vivo* tests required to establish benchmark doses, as well as increasing mechanistic understanding. However, matching target dose to target organ is an often overlooked aspect of *in vitro* assays, and the calibration of *in vitro* exposure against *in vivo* benchmark doses is often ignored, inadvertently or otherwise. An example of this was recently published in *Environmental Health Perspectives* by Wagner *et al* (2016), where neural stems cells were used to model the molecular toxicity of lead. On closer examination of the *in vitro* work, the doses used in media reflected *in vivo* lead doses that would be at the highest end of lead toxicity, perhaps even lethal. Here we discuss the doses used and suggest more realistic doses for future work with stem cells or other neuronal cell lines.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED version 2 published 30 Mar 2017		
version 1 published 29 Nov 2016	✓ report	✓ report

1 **Mir Ahamed Hossain**, Department of Neurology USA

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REVISED **Amendments from Version 1**

The content of the article has been modified in response to the reviewers, and further we have separately included our response to the reviewers of our correspondence article.

See referee reports

A recent article by Wagner *et al.* reported the involvement of the anti-oxidant Nrf2 transcription factor signaling pathway in the toxicity of lead using neural stem cells in an *in vitro* model of neuronal differentiation[1]. While this work was completed in a similar way to other studies involving *in vitro* lead exposure, the work avoids a critical, often neglected issue of what constitutes a relevant physiological dose *in vitro*. The assumption that the selected dose of 1 µM (or 20.7 µg/dL) for neuronal stem cell exposure was "4 times the CDC levels of concern (LOC) for blood lead (5 µg/dL) and is within the range of exposed populations" requires further exami-nation. Since the *in vitro* exposure was completed in media (the equivalent of plasma or serum) and not in whole blood, the assump-tion that the *in vitro* lead level would be equivalent to that found in whole blood of lead-exposed humans is somewhat inaccurate. Lead in serum (or plasma) represents only a fraction (~1%) of the level found in whole blood[2,3], with the major fraction of lead bound inside erythrocytes[4]. For arguments sake, if the proportion of lead used in this study was 1% of that in whole blood, the extrapolated blood lead value would be approximately 2073 µg/dL, a level over 400 times the CDC LOC, and one that would be acutely toxic and perhaps lethal.

Another study, which was cited by Wagner *et al.*[1], showed that measurable effects in stem cells *in vitro* could occur at doses as low as 0.4 µM[5]; this dose would represent a blood lead level of 829 µg/dL, using the same assumptions as above. In a study by Chan *et al.,* the lowest dose of 1 µM lead used in a study of newborn rat neuronal stem cells would represent 20.73 µg/L in serum and a systemic blood lead level of about 2073 µg/dL[6]. Other stud-ies examining the toxicity of lead in cell cultures have also failed to adequately match the *in vitro* doses[7–9] with those found *in vivo*, by taking account of the well documented relationship between plasma and whole blood lead values. More importantly, with measurable effects only beginning at greater than 10 µM for some studies[6,9], could these data suggest the alternative conclusion - that neuronal cells *in vivo* are more resistant to toxic insult by lead, at least in the short term?

What is clear is that at current blood lead levels in the US population, serum or plasma levels will represent a very low fraction of those values and *in vitro* work could more realistically model neurological effects in humans if target doses were better matched to target organ. Thus, the model of exposure proposed by Wagner *et al.* and other *in vitro* work demonstrating toxic effects of lead[5–9] may be more appropriate for high acute exposures. More realistically, to ensure that doses used for *in vitro* assays are complimentary to a target *in vivo* blood lead level of 20 µg/dL, exposure to cells *in vitro* should correspond to ~1% of the cited blood lead value, or a dose of 0.2 µg/dL (0.01 µM). At the current CDC 5 µg/dL LOC for children, the *in vitro* dose would become 0.05 µg/dL (0.002 µM); a dose that would present difficulties to laboratories that cannot eliminate background levels from residual lead on glassware and other sources of possible contamination or confounding of the reported data. Background contamination in controls would mean requiring higher exposure doses to demonstrate an effect, essentially making the assays less sensitive.

In the study by Wagner *et al.*[1], much of this may have been considered by the authors, and key assumptions may have been made; however, the question still remains whether the upregulation of genes in the Nrf2-mediated anti-oxidative stress pathway would have been observed if a more physiologically relevant dose of 0.2 µg/dL (0.1 µM) in the media (i.e., representing a blood lead level of 20 µg/dL) had been used.

How does lead in plasma compare to lead in cerebrospinal fluid? Presumably the plasma fraction contains the lead moiety that interacts with molecular targets in the brain. Evidence shows that lead in cerebrospinal fluid is 50% of that in serum[2], indicating that the assumptions made here are consistent with target doses of lead in the brain being much closer in value to plasma than to whole blood lead. We did not account of the evidence that the proportion of lead in plasma increases with increasing blood lead value[3,4] – which could affect our upward extrapolations from putative plasma values of 20 µg/dL to whole blood lead levels of 2073 µg/dL – but it should not affect extrapolating downward to plasma lead from a starting blood lead of 20 µg/dL as the relationship between whole blood and plasma lead seems to be linear in that region[3]. However, even if we used a value of 5% lead in plasma the extrapolated blood lead for the Wagner *et al.* study would turn out to be 20-fold the plasma which is 400 µg/dL.

Our article raises questions about what a relevant *in vitro* lead dose should be when it is contextually related to *in vivo* blood lead values. A scan of the literature for this article has shown that there are a significant number of *in vitro* publications using lead that lack (or even misinterpret) context with whole blood lead levels, thereby identifying molecular effects that may not have relevance to current national blood lead values. We propose that matching target dose to target organ should be more carefully considered with future *in vitro* work.

Disclaimer

The views expressed in this article are those of the author(s) and do not necessarily reflect the official policy of the Department of Defense, Department of the Army, U.S. Army Medical Department or the U.S.

Author contributions

DB conceptualized the article and analyzed the original critiqued article reported herein. MW provided technical writing support and analysis of the original critiqued article reported herein.

Competing interests

No competing interests were disclosed.

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Current Referee Status: ✔ ✔

Version 1

Referee Report 26 January 2017

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✔ **Donald Smith**
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This commentary is well written, very well justified, and timely. While there are countless published papers on the myriad effects of lead in biological systems, the consideration of dose extrapolation from *in vitro* to *in vivo* studies and their relationships to the human condition often goes unappreciated. Indeed, since toxicology is driven by the dose of the poison, establishing environmental or occupational relevance of the dose is absolutely key to the relevance of the findings. This commentary points this out in a concise and evidence-driven fashion, and is worthy of publication.

Below are a few minor comments to consider.

1. Pg. 2, 1st para: *For arguments sake, …*

   Comment: A caveat here might be that is known that the proportion of whole blood lead in plasma increases with increasing blood lead, so it is likely that the blood lead level that would produce a 1 uM plasma lead would be lower than 2,073 ug/dL, but this does not detract from the point the authors are making, which is a good and important one.

2. Pg. 2, 3rd para: *Thus, the model proposed in this and other work…*

   Comment: It is not clear whose work 'this work' is referring to - Chan *et al*?

3. Pg. 2, 3rd para: *To ensure that doses used in in vitro assays are complimentary to a target in vivo blood lead level of 20 µg/dL…*

   Comment: This suggestion by the authors is reasonable, assuming that plasma lead reflects extracellular fluid lead, though it might also be worth looking at the relationship between blood lead and CSF lead levels (in the literature) to see if it follows an appx 1% relationship as does plasma to further substantiate this suggestion.

4. Pg. 2, 3rd para: *…eliminate background levels from residual lead on glassware and other sources of possible contamination or confounding of the reported data…*

   Comment: This too raises an important point in that the vast majority of studies do not make sufficient effort to reduce background lead levels in control cultures, so it is quite possible that here

and in those other studies the control cultures, even with modestly elevated background lead levels will also be affected, requiring higher exposure doses to demonstrate a difference or 'effect' in the lead-exposed treatments. It is good that the authors pointed this out.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response (*Member of the F1000 Faculty* and *F1000Research Advisory Board Member*) 02 Mar 2017

Mark A Williams, Army Public Health Center, USA

Reviewer 2. We thank reviewer #2 for knowledgeable and helpful comments on our article. Here are our responses to specific comments.

Comment 1. This point is well made – we agree that the proportion of lead in plasma would increase as blood lead increases, so that equivalent plasma lead at blood lead values greater than 100 µg/dL could be upwards of 2%. As it was we selected 1% plasma/blood ratio as the blood lead under question was 20 µg/dL but of course there is some inbuilt error in our calculations at high doses. Nonetheless, our extrapolated exposure scenario is meant to demonstrate that the assumptions under which many *in vitro* studies lie with respect to their relationship to *in vivo* blood lead values are often violated; the reviewer also acknowledges our efforts to point this out. We have added more text to acknowledge this non-linear relationship at increasing doses between whole blood lead and plasma lead.

Comment 2. This sentence has been restructured to indicate that we referring to the Wagner *et al* study, as well as other studies that have made similar assumption.

Comment 3. We agree that cerebrospinal fluid measures would further corroborate our assumptions. The work by Manton *et al* (cited in our article) showed that cerebrospinal fluid levels were about 50% of serum levels, though it should be pointed out that this work was carried out in only one subject. We have added more text to acknowledge this fact.

Comment 4. We agree with the further elaboration of this sentence and have added additional text to incorporate the details of the comment.

Competing Interests: None.

Referee Report 18 January 2017

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Mir Ahamed Hossain
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In vitro assays have become a mainstay of modern approaches to toxicology with a high promise of understanding the underlying mechanisms of toxicity. The results reported by Wagner *et al.*, (2016) in the August 26 issue of the Environmental Health Perspectives, where neural stem cells were used to model

the toxicity of lead. The results support the notion that lead treatment of cells leads to upregulation of vascular gene expression (JBC 275:27874-27882, 2000). While this work presents interesting effects, this reviewer's opinion is in agreement with the correspondence (critiqued article) authors Bannon and Williams that it may be more appropriate for high acute exposures particularly in case of neural stem/progenitor cells, which lack many of the characteristic features of mature neurons.

It is also likely that neural stem cells (NSCs) could be more resistance to toxic insult by lead - at least in the short term. Thus the *in vitro* work could more realistically model chronic neurological effects if doses are better matched with the doses at the target site, as supported by the fact that serum or plasma levels represent a very low fraction of the total blood lead levels. Thus the concentrations of lead used in this study, which elicits upregulation of genes in the Nrf2-mediated anti-oxidative stress pathway, appear to be in the low micromolar range, which is much higher than the *in vitro* dose equivalent of the current CDC levels of concentrations (5 ug/dL) for children. Thus the concentrations used in the study does not reflect the likely exposure of lead in the environment, that is to say, concentrations which are likely to be cytotoxic particularly in case of NSCs. This is clearly a near impossible issue to address empirically, but if some information available along these lines using a more physiologically relevant dose in the media of *in vitro* NSC cultures to show gene expression in the Nrf2-mediated anti-oxidative stress pathway would be helpful for the reader as suggested by Bannon and Williams in the critiqued article. It will also be interesting to see how the differentiated neurons from lead exposed NSCs express neurons specific features or exhibit mature neuronal function.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response (*Member of the F1000 Faculty* and *F1000Research Advisory Board Member*) 02 Mar 2017

Mark A Williams, Army Public Health Center, USA

Reviewer 1. We thank reviewer #1 for helpful comments on our article. We address some specific aspects below.

The reviewer agreed with our principle argument, but goes on to state that the Wagner *et al* "results support the notion that lead treatment of cells leads to upregulation of vascular gene expression", citing an *in vitro* microarray study using astrocytes, (Hossain *et al*, 2002, ref 9 above) when in fact two of the three VEGF transcripts listed in Supplemental Table 1 of Wagner *et al* were downregulated by lead, with only one – VEGFA downregulated by 0.8-fold – being statistically significant. Therefore the cited publication by Hossain *et al* is contradicted by the Wagner *et al* data for the VEGF gene.

The fact that the Hossain *et al* study used 10 µM lead acetate to dose astrocytes *in vitro* further supports our main point – that most lead concentrations *in vitro* would reflect highly lethal lead concentrations *in vivo* if the difference between lead in whole blood (red blood cells) and plasma were taken into account. Hossain *et al* did cite Audersirk (Audesirk G, *et al.* In Vitro Cell Dev Biol. 1989 Dec;25(12):1121-8) as supporting evidence for the use of 10 µM lead as a dosing solution for astrocytes, where Audersirk measured free lead (Pb^{2+}) in the nanomolar range in the presence of full experimental media dosed with micromolar lead acetate using an ion selective electrode. However, Audersirk's work in snail and chick neurons did not examine the potential lethality of the *in vitro* working doses to the whole organism, taking account of plasma/whole blood differences.

Competing Interests: None.