Author’s response to reviews

Title: Toxicity of insulin-derived amyloidosis: a case report

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Alexander Kokkinos
Associate Editor
BMC Endocrine Disorders
Dear Dr. Kokkinos:

Thank you for the consideration you have given our manuscript entitled, “Toxicity of insulin-derived amyloidosis: a case report”.

We are grateful to you and the reviewers for the valuable comments. We have made revisions according to the comments, and the responses are included at the bottom of this letter. Especially, in the process of re-evaluating the statistical analyses, we performed additional toxicity assays, and therefore updated Figure 3.

We believe that the revised manuscript is improved over the original version and hope that it will now be acceptable for publication in BMC Endocrine Disorders.

Sincerely yours,

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Responses to Editor and Reviewers' Comments:

To the Editor and Reviewers

--Thank you very much for your valuable comments and advice. Based on your advice regarding the statistical methods for toxicity studies, we performed one-way analysis of variance (ANOVA), instead of Student’s t-test, for original triplicate data. This analysis showed that statistical significances were maintained in the HEK293T cell assay and were lost in the HeLa cells assay.
We also performed additional toxicity studies using triplicate samples with HEK293T and HeLa cells, resulting in obtaining two sets of triplicate data. We then used one-way ANOVA for comparison among the samples from Cases 1-5 and the control. After a significant difference was obtained by one-way ANOVA, the post hoc Tukey test was performed. The results showed that two samples (Case 5-1 and 5-2) in the HEK293T cell assay and one sample (Case 5-2) in the HeLa cell assay had statistically more significant toxicity than the control or the other four samples from Cases 1-4.

Therefore, we changed the manuscript text (Page 9 Lines 6 – 16) and the figure legend for Fig. 3 (Page 19), and updated Fig. 3.

To Dr. Hiraku Kameda (Reviewer 1)

In this paper, the authors describe a case of insulin-derived amyloidosis with surrounding necrotic tissues and its cell toxicity. Although they have already described the clinical course of the cases in "Am J Med. 2014 May;127(5):450-4.", in this paper they showed additional histological and in vitro studies.

This paper is generally well-written and may have a clinical significance, implying the necessity of further investigation for toxic insulin-derived amyloidosis.

--Thank you very much for your supportive comments. We agree with you that further investigation of toxic insulin-derived amyloidosis is necessary.

To Dr. Akinobu Nakamura (Reviewer 2)

Authors described the case of toxic insulin-derived amyloidosis. Histological and immunohistochemical studies showed that the mass had typical characteristics of amyloid and necrotic findings were seen adjacent to the amyloid deposit in this case. Furthermore, the amyloid tissue of the present case showed significant toxicities against both HEK293T cells and HeLa cells compared to the control skin tissue. This case report is interesting in essence. There are, however, some points which should be addressed for publication in this journal.

Specific comments

Criticism 1: Please describe the type of insulin preparation in Table 1.
Thank you very much for your advice. We described the type of insulin preparation in Table 1.

Criticism 2: Please provide the photographs of skin findings.
--We agree with your comments. However, we do not have the photographs of the skin findings.

Criticism 3: In the toxicity studies, Student’s t-test is inappropriate.
--Thank you very much for your advice. As described above, we used one-way ANOVA instead of Student’s t-test.

Criticism 4: "Additionally, the two samples from Case 5 showed more significant toxicity than the four other samples." Are there any significant differences in cell viability between the two samples from Case 5 and the other four samples (Case 1-4) both in HEK293T cells and HeLa cells?
-- As described above, two samples (Case 5-1 and 5-2) in the HEK293T cell assay and one sample (Case 5-2) in the HeLa cell assay had statistically more significant toxicity than the other four samples from Cases 1-4 on the post hoc Tukey test after one-way ANOVA.

To Dr. Kabirullah Lutfy (Reviewer 3)
The current manuscript is a case report where in five diabetic patients receiving insulin treatment for 12 - 25 years, the authors found amyloidosis at the site of insulin injection. Among the five cases studied, all subject had this type of condition; however, only case 5 who was a younger male with one of the leg amputated had a form of toxic amyloidosis since the authors showed that it caused cell death in both types of cell used compared to control group in which tissue was taken from the dermis, epidermis and subcutaneous tissues. Overall, the paper is well written and the results are of interest to the readers of the journal. However, there are issues that need to be addressed.
Major:

1. There is no control group for each case and it is not clear what makes the control group. Is it pooled data for all cases or just all were compared against control of case 5?

   --Thank you very much for your valuable comments. The control consisted of microdissected tissue including epidermis, dermis, and subcutaneous tissue. It was pooled tissue from Cases 1-5 because the amount of tissue from one case was small. The main text (Page 8 Lines 13 – 16) was changed to clarify this.

2. The duration of insulin treatment, the sex of subject and their age vary in some cases significantly (58 years vs. 82 years). However, the authors did not discuss this as a factor affecting the result. Importantly, the dose of insulin and effectiveness insulin in each patient should be provided and discussed so the readers can have a better idea.

   --We agree with you that clinical characteristics are important factors. However, we think that a discussion of clinical characteristics on the basis of a small number of patients might be misleading. For example, Case 5 had type 2 diabetes and Cases 1-4 had type 1 diabetes, implying that type 2 diabetes may be associated with necrosis or toxicity in this setting. However, we had 9 other patients with insulin-derived amyloidosis who had type 2 diabetes, but they did not have necrosis around the amyloid deposits. Therefore, we compared the clinical characteristics between Case 5 and the other 15 cases, including the four cases in this study, and we found that the clinical characteristics were similar in Case 5 and the other cases. We described these findings in the main text (Page 12 Lines 8 – 18) and updated Table 1.

3. The data seem to be a candidate for one-way ANOVA not student's t test.

   --Thank you very much for your advice. As described above, we used one-way ANOVA, instead of Student’s t-test, as you suggested.

4. Were necrotic tissues cleaned when the materials collected for each case? Would the presence of necrotic tissue cause toxicity to these cells?

   --Necrotic tissues were not cleaned after skin incision biopsy, and a drainage tube was therefore put into the necrotic tissue for discharge of pus.

   The idea that the presence of necrotic tissue causes toxicity of the amyloid deposits is very interesting, and we cannot entirely rule out this possibility. On the other hand, we could not find
other causes of necrosis on histological examinations and clinical findings. Therefore, we think that the toxicity of amyloid deposits may cause necrosis in the surrounding tissue.

Minor

If possible, have a legend for figure 1, please include case 2 and case 5 on the top for A-D and case 5 for E-H and on the left or right of each panel, Congo Red, Polarized Field, Insulin immunostaining and amyloid protein from the top to the bottom, respectively.

--Thank you very much for your advice. We changed Fig. 1 accordingly.