

February 13, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 15339-review.doc).

Title: Contrast-Enhanced Micro-Computed Tomography Using ExiTron nano6000 for the Assessment of Liver Injury in Mice Models

Author: Xiangwei Hua, Tianfei Lu, Dawei Li, Weigang Wang, Jun Li, Zhenze Liu, Weiwei Lin, Jianjun Zhang, Qiang Xia

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 15339

We revised the manuscript in accordance with the reviewers' comments, and carefully proof-read the manuscript to minimize typographical, grammatical, and bibliographical errors. We highlighted the changes by using the text on green background in our manuscript.

Here below is our description on revision according to the reviewers' comments.

Part A (Reviewer 1)

The reviewer's comment: This is very interesting paper. Author conclude that CECT was able to objectively detect liver lesions based on the texture and density alterations of the CECT image; these alterations were caused by variations in macrophage distribution, number and function. I ask some questions. ①Please tell me the reason why there are no macrophage in LPS/D-GalN - induced hepatic necrosis in CD68 immunostained (Fig 3B). ②Please tell me the reason why macrophages were not present in the area of either the bile infarct or the dilated biliary tract. ③Please tell me the reason why CECT predominantly shows the area rich in macrophage.

The authors' answer: ①Owing to the widespread histolysis, macrophage decreased in the liver tissue. So we see the scarce CD68 staining in LPS/D-GalN induced hepatic necrosis. ②There are no cells existing in the area of either the bile infarct or the dilated biliary tract. The macrophages may be surrounding them. ③High soft tissue contrast is necessary to detect liver disease in small animals for micro-CT imaging. ExiTron nano6000 is a contrast agent, which provides a positive contrast of the liver. CECT only show the area abundant with ExiTron nano6000. And ExiTron nano6000 was mainly taken up by macrophage. So CECT predominantly shows the area rich in macrophage.

Part B (Reviewer 2)

The reviewer's comment: In this article, the author showed that Micro-CT with the contrast agent ExiTron nano6000 is useful for detecting various liver lesions such as BDL, LPS/D-GalN and alcoholic liver injury by emphasizing the heterogeneous textures and densities of CECT images, which were due to the distribution, number, and function of macrophages. These results are novel and very interesting. Major: ①The picture of Fig2, especially LPS/D-GalN (PBS, 3h, 6h), is not representative, because it is very difficult to find CD68 positive cells. These figures must be the key figures in this article, which allow the author to show the association between macrophage activation and uptake of the contrast medium, so the author should fix this problem definitely. According to the previous articles (for example; J Hepatol. 2014 May; 60:1032-9 PMID: 24412604). The different antibody of CD68 (FA-11, Serotec) worked decently for staining macrophages in mouse liver. ② Author showed the RAW246.7 cell mass presented a significantly increased density on the CECT images after co-incubation with ExiTron nano6000, which supported the ability of macrophages to uptake this contrast agent in vitro. Is there any other ways to show that macrophage activation accelerated uptake of the contrast medium in vivo liver tissue injured by LPS? Also, are there any methods to show macrophages phagocytize the

particles of ExiTron nano6000? If doable, it makes this paper more decent.③According to the H&E staining in Fig 3, Liver tissue showed strong hepatocytes necrosis 6h post treatment of LPS, which might be the main cause by which the uptake of agent ExiTron nano6000 was reduced at 6h. Therefore, hepatocytes might be main cells absorbing the contrast medium more than macrophages. For excluding this possibility, author required to evaluate the influence of hepatocytes on ExiTron nano6000 uptake under the LPS treatment Minor. ①What is the meaning of “The black arrows indicate black regions.” in fig legend for Fig4?

The authors’ answer: major ①We have tried our best to modify the pictures in revised manuscript. Besides, in our study, we can see the representative CD68 positive cells in BDL-treated mice. But in LPS/D-GalN-induced liver lesions, the CD68 stain was not so much good. It may be due to the different expression of CD68 in different models. ②In the first part of results, we found the increased liver densities in the CECT images after liver lesions. In the late parts, we try to explain the phenomenon. Here, we firstly investigate the influence of macrophage number to the CECT densities. Then, we try to further explore the problem from the angle of macrophage function. Although we didn’t find the relative studies to solve the problem, we performed a vitro study and explained the phenomenon in a reasonable way. Until now, we are sorry that we have not found other ways to prove it. We believe we can solve the problem in the future with the progress of technology.③At first, just like macrophages, we did perform CECT images of hepatocytes. But we did not find that hepatocytes could uptake Exitron nano6000, which is according with the product specifications of Exitron nano6000 and previous studies(PLoS One 2011;6:e25692 PMID:21984939; Acad Radiol 2013;20:1137-1143 PMID: 23931428). **minor** ①The black arrows indicate black regions with low densities, which refer to necrosis or dilated biliary tract. We have revised the description in the manuscript.

Part C (Reviewer 3)

The reviewer’s comment: Contrast-Enhanced Micro-Computed Tomography Using ExiTron nano6000 for the Assessment of Liver Injury in Mice Models by Xiangwei Hua and coauthors This is a well-done, thoughtful manuscript that is well written. The authors showed that Micro-CT with the contrast agent ExiTron nano6000 is potentially useful for detecting various liver lesions such as for example alcoholic liver changes by the heterogeneous textures and densities images, depending on the distribution, number, and function of macrophages. As a reviewer, I also believe that it has the potential to provide important information about the importance of new technique for detecting different liver lesions. However, there are a few questions I have about the study. Numbered comments: ①Micro-CT with the contrast agent ExiTron nano6000 was applied to assess only three types of liver lesions. Are there any other liver lesions- potential candidates for this type of imaging? ②Describe the special characteristics of ExiTron nano6000 contrast agent, which provide possibility to be used for detection of liver lesions. ③Macrophages are present also in other tissues. Are there any studies published about the possible use of this technique for detecting lesions in other organs?

The authors’ answer: ①In the fact, we also performed the assessment for APAP induced liver lesions. The result was similar to LPS/D-GalN induced liver lesion. Besides, a previous study also proved ExiTron nano6000 facilitate the detection of liver tumors. We think the method could be used to detect any liver lesion that has changed the macrophages in distribution, number or function. Here, we just choose the three typical models related to the changes of macrophages.②ExiTron nano6000 is an alkaline earth metal-based nanoparticulate contrast agent specifically formulated for preclinical computed tomography (CT). It shows strong X-ray absorption due to the high metal load of the particles. Upon intravenous injection, ExiTron nano6000 circulates in the blood stream and accumulates particularly in the liver and spleen and provides long-term X-ray contrast. ExiTron nano6000 is primarily taken up by cells in the RES (reticuloendothelial system), including macrophages, which are distributed extensively in the liver as Kupffer cells.③No. But previous study (PLOS One 2011;6:e25692 PMID:21984939) has pointed that ExiTron nano6000 also provides strong contrast of lymph nodes and adrenal glands, hereby allowing longitudinal monitoring of pathological processes of these organs in small animals.

Thank you and all the reviewers for the kind advice.

Sincerely,

A handwritten signature in dark ink, appearing to read 'Xia Qiang', with a stylized flourish at the end.

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