Peer Review File

Article Information: http://dx.doi.org/10.21037/cdt-20-609

Review Comments:

The purpose of the study is to track the development of vascular inflammation by measuring FDG and fluoride uptake in ApoE -/- rats fed a "western" diet. The authors presented SUV values at the aortic arch for FDG PET and SUV values at the pulmonary artery for fluoride PET at 3 different time points. The authors also presented histologic analysis at the aortic arch and the pulmonary artery.

The use of fluoride and FDG PET for longitudinal study of inflammation in rat animal model for atherosclerosis is very logical. However, the study design and data as presented have major problems.

Major issues:

1. No negative control for imaging: The authors employed very reasonable control for biochemical examinations which demonstrated significantly different cholesterol, LDL and HDL values between the ApoE -/- rats and the control rats. However, there is no negative control for the imaging portion of the study. Although three Sprague-Dawley rats were used for control, no imaging were performed on these animals. The authors cannot rule out the possibility that the SUV values can be normal age related progression in wild type (SD) rats. The authors have not proved that the SUV valued measured on the ApoE -/- rats are statistically different from potential measurements on wild type rats.

Response:

We sincerely appreciate the valuable suggestion for improving our manuscript. In this study, we did perform PET/CT imaging for control rats. But for control rats at 12 weeks of age, there was [^{18}F]FDG uptake in thymus gland which might be physiologic uptake in young rats and is very common in human [1]. The previous analysis method

was subjective that it was difficult to discriminate the aortic arch from the other tissues by CT images. In this revised manuscript, we used T1-weighted MRI images for atlas template and analyzed atlas-based VOIs (from page 8, line 18 to page 9, line 6; Figure 3). Though the [^{18}F]FDG uptake in the aortic arch of normal rats at 12 weeks of age was still affected by the spill-out effect of the other tissues, the location of the artery was more accurate and objective. We compared the tracers' uptakes in the control group and the Apoe group. It showed that for the [^{18}F]FDG uptake in the aortic arch at 46 weeks of age and the [^{18}F]NaF uptake in pulmonary arteries at 27 and 46 weeks of age, the uptake in the Apoe group was significantly higher than in the control group (page 11, line 1-14; Figure 4B, C).

Reference:

1. Ferdinand B, Gupta P, Kramer EL. Spectrum of thymic uptake at 18F-FDG PET. Radiographics. 2004;24(6):1611-1616.

2. No negative control for histology. The authors demonstrated significantly increased HIF-1-alpha expression in the aortic arch compared to the LPA. However, without a negative control, one cannot conclude that HIF-1-alpha expression is abnormal. The authors have not excluded the possibility that there is increased HIF-1-alpha expression in the aortic arch compared to the LPA in wild type rats. Similarly, microcalcification and CD68 expression in the pulmonary arteries may be elevated compared to microcalcification and CD68 expression in the aortic arch in normal rats.

Response:

We sincerely appreciate the valuable suggestion. In the revised manuscript, we also provide the histology result of the control group (page 12, line 2-11; Figure 5). It is more persuasive when comparing the positive results in the Apoe group with negative results in the control group.

3. **Imaging experimental design is possibly flawed. The beauty of using microPET to survey the cardiovascular system is that the entire system can be surveyed**

longitudinally. The authors devoted a lot of resources to acquire the whole body images of the rats, but the authors only presented SUV values from 1 location for each tracer. The article as written is not a representative of the dataset that was acquired by the authors. The authors stated in the method section that "hot spots" around the aortic arch and the pulmonary arteries were measured. The authors need to clearly defined what a "hot spot" is. The authors also need to clearly define how ROI's are drawn. Based on the description provided by the authors, the ROI may be different from rat to rat depending on where the "hot spots" are; this can lead to measurement bias.

Response:

We agreed that the previous analysis method of just drawing ROIs around hot spots was biased. Only using non-contrast CT to depict the vascular anatomy is difficult. We presumed that atlas-based VOI analysis, which is commonly used in brain research, could be useful in this research. To revise the manuscript, we acquired T1 weighted MRI images of a normal SD rat. The aortic arch and pulmonary arteries in MRI images are very clear. Then we drew the VOIs of the aortic arch and pulmonary arteries and used it as an atlas template. At last, we analyzed all the data by manual co-registration of MRI to PET/CT and assessment of tracers' uptakes in atlas VOIs (from page 8, line 18 to page 9, line 6; Figure 3).

In this study, though we acquired the whole-body PET/CT image of rats, it is still difficult to locate arteries. The $Apoe^{-/-}$ rats we used did not develop extensive atherosclerosis plaques as we expected except in pulmonary arteries. In the end, we only analyzed the aortic arch and pulmonary arteries.

4. The authors should clarify the choice of using the aortic arch for SUV measurements. Previous publication regarding ApoE knock-out rats using TALEN technology (J Biomed Res. 2017 Jan; 31(1): 47–55, PMID: 28808185) indicate that there is mild atherosclerosis throughout the aorta. There is no indication that the aortic arch having unique pathology compared to other segments of the aorta. It would be unusual for FDG uptake related to

atherosclerotic disease to be observable only in the arch. The reported images are highly cropped and windowed such that a reader cannot clearly appreciate the overall physiologic activity nor the location of the focal activity. In the presented images, the FDG uptake appears to be within the anterior mediastinum, and could have been thymic FDG activity. The authors should submit supplemental data with maximal intensity projection (MIP) whole body images and high resolution axial view and oblique reformat images to demonstrate that the FDG uptake is truly restricted to the walls of aortic arches.

Response:

We agree that the aortic arch does not have unique pathology compared to other segments of the aorta. The [^{18}F]FDG may not only accumulate in the aortic arch. Here we only analyzed the aortic arch because it is easy to locate in images. We have made some modifications to diminish the ambiguity.

Gao et al and some researchers reported the formation of mild atherosclerosis in the aorta [1,2], others reported no obvious atherosclerotic lesion was see in the aorta [3,4]. In our study, we found there was no obvious atherosclerosis in the aorta. Because aortic arch is easy to identify in images, we analyzed the aortic arch in this study rather than other segments in the aorta. The purpose of this study would be to find the correlation of inflammation and calcification in an atherosclerotic plaque rather than discover atherosclerosis in all arteries. But the result is beyond our expectation.

Even though we could submit supplemental data, with non-enhanced CT images, it is still difficult to locate the [^{18}F]FDG uptakes inside the walls of aortic arches. Atlas analysis based on T1-weighted MRI might be an alternative method as mentioned earlier.

Reference:

1. Gao M, Xin G, Qiu X, Wang Y, Liu G. Establishment of a rat model with diet-induced coronary atherosclerosis. J Biomed Res. 2016 Oct 17;31(1):47-55.

2. Rune I, Rolin B, Lykkesfeldt J, Nielsen DS, Krych Ł, Kanter JE, Bornfeldt KE, Kihl P, Buschard K, Josefsen K, Fels JJ, Mortensen A, Christoffersen B, Kirk RK, Hansen AK. Long-term Western diet fed apolipoprotein E-deficient rats exhibit only modest

early atherosclerotic characteristics. Sci Rep. 2018 Apr 3;8(1):5416.
3. Wei S, Zhang Y, Su L, He K, Wang Q, Zhang Y, Yang D, Yang Y, Ma S. Apolipoprotein E-deficient rats develop atherosclerotic plaques in partially ligated carotid arteries. Atherosclerosis. 2015 Dec;243(2):589-92.
4. Phillips EH, Chang MS, Gorman S, Qureshi HJ, Ejendal KFK, Kinzer-Ursem TL, Blaize AN, Goergen CJ. Angiotensin II Infusion Does Not Cause Abdominal Aortic Aneurysms in Apolipoprotein E-Deficient Rats. J Vasc Res. 2018;55(1):1-12.

4. The authors stated that "…our results showed that [18F]FDG PET imaging failed to visualize inflammation in early atherosclerotic lesions…" The authors could be right; however, the data from 1 location presented by author is not sufficient to prove that statement. Also, a proper negative control (with images of wild type rats) is needed to demonstrate that FDG PET imaging did not visualize inflammation.

Response:

We sincerely appreciate the valuable suggestion. We should have provided PET/CT and pathology result in the normal group. Now we know the importance of the normal group and provide these data in the revised manuscript as suggested.

Minor issues:

1. As stated on page 9: 17 Apoe -/- rats and 3 Sprague-Dawley rats were purchased for experiment. On page 9, Apoe -/- rates (n=19) underwent PET/CT. The authors should clarify the discrepancy.

Response:

We sincerely thank the reviewer for careful reading. We have rechecked and corrected the number of rats. In the Apoe group, 19 Apoe$^{-/-}$ rats underwent PET/CT imaging, and aortic arch and pulmonary arteries from17 Apoe$^{-/-}$ rats were collected for the pathology study. The arteries of the other 2 Apoe$^{-/-}$ rats were cut and used for taking photos under a surgical microscope.

2. The population is very heavily weighted toward the experimental group. The authors should explain if there is a specific reason for the need for significantly larger population of the experimental group compared to the control group.

Response:

When we planned for this study, we thought there would be some death rate for these Apoe$^{-/-}$ rats. Because these Apoe$^{-/-}$ rats were shipped from the place very far away and would be fed with a high-fat diet for around a year. We bought an excess number of these rats than needed. In the end, by very careful care, all the Apoe$^{-/-}$ rats survived.

3. Of the 17 rats at week 12, LPA data came from 7 rats and RPA data came from 4 rats. All rats have LPA and RPA, all data points should be reported unless there is a clear explanation for exclusion of data. Selective measurements leads to bias in data.

Response:

We sincerely appreciate the valuable suggestion. In the revised manuscript we analyzed the atlas-based VOIs by software in both the left and right pulmonary arteries rather than if we could see it or not.