A Preliminary Investigation of Photoacoustic Imaging to Detect Lupus Nephritis

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Abstract

Lupus nephritis is renal disease caused by systemic lupus erythematosus. Renal biopsies are required in order to confirm lupus nephritis, an invasive procedure to the patient. The aim of this study was to detect and confirm lupus nephritis using a non-invasive procedure. This research tested the ability of photoacoustic imaging to detect disease activity and progression of glomerulonephritis (renal disease similar to lupus nephritis, but not caused by lupus) infected kidneys. Results suggest that photoacoustic imaging has limited ability to detect disease activity and progression because the technology is so new to the rheumatology field. Finding a non-invasive procedure that can be used to detect and confirm lupus nephritis will be highly beneficial to the patients' health as disease activity could be detected sooner, and more often, with no effects relative to an invasive procedure.

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease characterized by the immune system attacking healthy tissues of the body including the skin, joints, kidneys, heart, lungs, and brain. For many patients, uncertainty and lack of group support with their disease is very common because there is no known cause or cure (Cleanthous et al. 2013). Because there is very little known about SLE, doctors and researchers continue to advance their studies in the field every day.

Renal disease is one of the most common, and serious, effects that can come with having SLE. Inflammation of the kidney is called glomerulonephritis (GN), but when it is caused by SLE it is called lupus nephritis (LN). Patients often have a urine test with each doctor's visit, and if renal disease is detected, a biopsy of the kidney usually will follow (Norby et al. 2017). A renal biopsy is currently the only way to confirm kidney disease in human LN, as urine tests are not always accurate (Austin, 1998). Once a person is diagnosed with LN, they will typically have to get more than one biopsy to check the severity of the disease, or to make sure it has not returned once the patient is in remission. Patients in remission have between a 27% and 66% chance of relapsing, making continued biopsies important and necessary for the patients' health (Narvaez et al. 2017). Because biopsies are invasive to the body, there are a number of potential

risks including: bleeding of the biopsy site, blood in the urine, inability to urinate, and kidney infection (Kim, personal communication June 2019). In order to eliminate these risks, rheumatologists want to find a new way to detect kidney disease in lupus patients.

Photoacoustic imaging (PAI) is an emerging technology in the medical field that has been previously used to detect cancer and may be beneficial to LN patients. This technology works on the premise that it converts photons into ultrasonic waves to produce an image of deep tissues and organs of interest using different colors (Liu and Qin 2017). This type of imaging has shown to successfully detect disease deep into tissue in of various living organisms (Liu and Qin 2017). In recent years, PAI has been used to detect and monitor the growth of different types of cancers. Lee et al. (2017) used photoacoustic technology to monitor carcinoma growth and oxygen saturation of a tumor. Furthermore, the technology has been used to detect prostate cancer *in vivo* with its benefits being minimal invasiveness and real time imaging (Yang and Xiang 2017). With all of these recent advancements, PAI could potentially be used to detect LN in patients. Being able to detect LN using PAI would allow patients to get checked regularly without having to be biopsied each time.

According to our hypothesis PAI will be able to detect disease activity, and the progression of GN in the infected kidneys. We induced GN in several different mice, and used PAI technology to compare their diseased kidneys to the kidneys of control mice. Analysis of urine samples was performed for indication of GN.

Materials and Methods

Mice:

129X1/SvJ female mice (Jackson Laboratories, Bar Harbor, ME) and CD2AP knockout mice (gift from Jeffrey Miner, Washington University School of Medicine), between 6 and 10 weeks old, were kept under specific pathogen-free conditions in the Washington University School of Medicine animal facilities following. The NIH Guide for the Care and Use of Laboratory Animals, Use of Animals in Department of Defense (DoD) Programs, The Care and Use of Laboratory Animals in DoD Programs, and Washington University School of Medicine animal care guidelines were followed (approval #20160290, approval date: 03 February 2017, expiration date: 03 February 2020). Standards from the United States Army Medical Research and Material Command Animal Care and Use Review Office were also followed (approval date: 16 March 2017). This species of mice was chosen because they have a history of being involved in

successful research having to do with kidney diseases. In our lab they were fed regular flow chow, always had water available, and did not go through any specific light and dark cycles.

Nephrotoxic nephritis model:

Sheep anti-rat glomerular basement membrane (anti-GBM) serum was obtained from Probetex, San Antonio, TX. 100 μL of anti-GBM serum was injected through the tail vein in the mice to induce glomerulonephritis (GN). Two mice were injected with serum, while two mice served as the control in this preliminary study. Imaging was done at days 0 (baseline), 1, 4, 7, 10 (early GN),14, and 21 (late GN).

Proteinuria:

Urine samples from each mouse were collected passively in containers and pipetted into Eppendorf tubes on days 0, 1, 4, 7, 10, 14, 21 (with some exceptions of missed samples), and placed in a -20 C freezer for storage. For analysis, 16 uL of each urine sample was transferred to a new Eppendorf tube along with 4 uL of 5x Laemmli sample buffer. A positive control Albumin sample was also made using 2 uL of BSA, 14 uL of biograde H_2O, and 4 uL of 5x Laemmli sample buffer. All samples were heated for 5 minutes at 95 C. Urine samples were loaded into a 12.5% acrylamide SDS-PAGE gel along with a ladder and positive control of Albumin. The gel was run for 40 minutes at 160 volts. The gel was stained using Stains A, B, C, and D, with Stain D remaining in the container overnight. Stain A was composed of 25 percent Isopropanol, 10 percent Acetic Acid, and 0.05 percent Coomassie Brilliant Blue R-250. Stain B was composed of 10 percent Isopropanol, 10 percent Acetic Acid, and 0.005 percent Coomassie Brilliant Blue R-250. Stain C was composed of 10 percent Acetic Acid, 0.002 percent Coomassie Brilliant Blue R-250. Stain D was composed of 10 percent Acetic Acid. For each stain the rest of the 100 percent was made up of deionized water. The next day, proteinuria was confirmed by comparing mouse sample bands to the positive control albumin bands at 70 kDa.

MSOT imaging:

Mice were imaged using the Multispectral Optoacoustic Tomography (MOST) inVision 256-TF imaging system (iTheraMedical GmbH, Munich, Germany). The mice were isoflurane-anesthetized for about 20-40 minutes at a time, placed on a thin, clear plastic, covered in ultrasound gel, wrapped in an airtight bag, and placed within the imaging chamber filled with distilled water. An excitation signal was generated by the system using visible and near-infrared wavelengths (nm): 700, 730,760,780, 800, 850, 900, 1065, 1150, and 1210. These wavelengths

detected lipid content, hemoglobin oxygenation, and hemoglobin deoxygenation of the kidneys. An acoustic signal was generated through vibration of the tissues caused by the excitation from the different wavelengths. This acoustic signal was automatically detected by the system. The viewMSOTTM software system (iTheraMedical GmbH) automates the imaging and post-processing analyses, which includes image reconstruction, spectral unmixing, which allows the program to differentiate between each signal, and quantification. SO_2 measurements were assessed using MatLab by identifying the kidney as the region of interest, and then using code to generate histogram values. Mean and median values were then quantified using the histograms.

Analysis:

Data from MatLab was analyzed statistically using R Studio. Two regression analyses and a student's t-test were run in order to determine any significance between control and experimental mice, and over time in a single group.

Results

Proteinuria:

SDS-PAGE was run in order to determine proteinuria. Figure 1 shows the results of the SDS-PAGE. The ladder is shown to indicate where 70 kDa is located, and the BSA is the positive Albumin control. The control mice show little to no Albumin in the urine (which is normal), while the NTS mice are highly proteinuric beginning at day 10, which is early GN. NTS mouse 2, day 4 urine did not show up on the gel, which was suspected to be because the urine was very "sticky" indicating proteinuria.

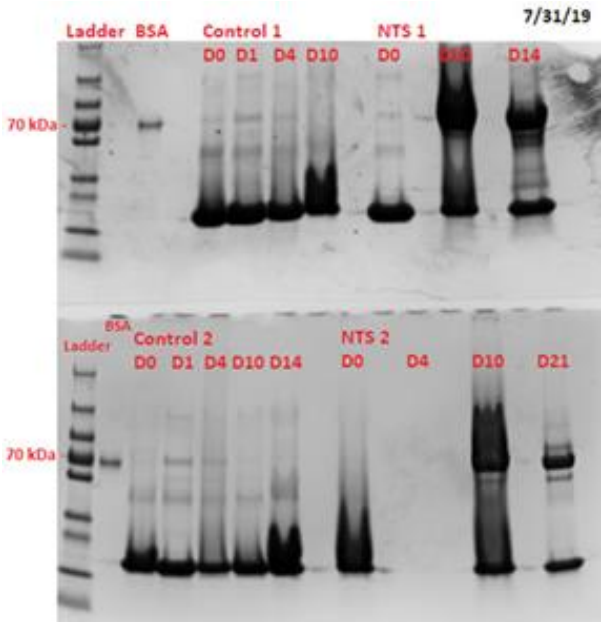

Figure 1. The ladder and BSA lanes in each gel are used as guidelines to show where the protein of interest, Albumin, is located. Control 1 is compared to NTS 1 in the top gel and Control 2 is compared to NTS 2 in the bottom gel. Control mice show little to no protein in the urine (normal), and NTS mice are highly proteinuric by day 10 as indicated by the band at 70 kDa.

MatLab Histogram analysis:

MatLab was used in order to select the kidney as the region of interest using the images created by the MSOT. This software then used code to determine the SO_2 value of each pixel in the kidney region. Each SO_2 value was then compiled together to make a histogram to represent each kidney slice. A mean value was determined from each histogram and recorded into a spreadsheet to use for statistical analysis.

R Studio analysis:

For each group of mice, a regression analysis was run in order to determine if SO_2 values in the kidneys changed over time. For the control mice there was a p value of 0.239, and for the NTS mice there was a p value of 0.110. These indicate no significant change in SO_2 in the kidneys over time. The results of the control group and NTS group regression are displayed in figures 2 and 3. A regression was then run to determine if there was any significant difference between each group of mice over time. The p value for this regression was 0.7325, which indicated no significant difference between mice groups over time, which can be seen in figure 4. Since no significant difference was found between the mice groups over time, all data points can be treated the same, so a student's t-test was run in order to determine any significant difference between the means of the control mice and NTS mice (figure 5). The p value of this test was 0.227, indicating no significant difference.

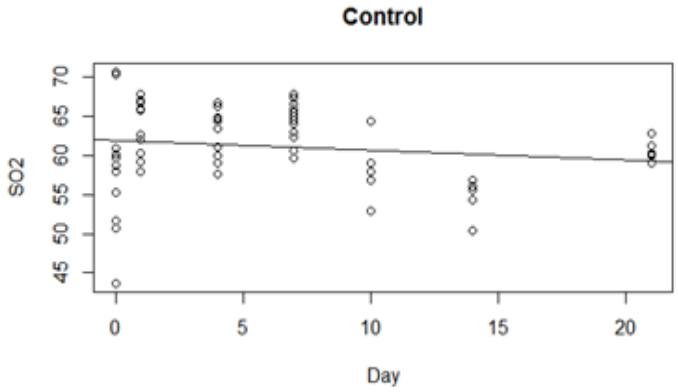

Figure 2. Regression analysis for the control data set. Day is displayed on the x axis and SO_2 values are displayed on the y axis. No significant change was found over time. A trendline is shown to see the tendency of the data over time.

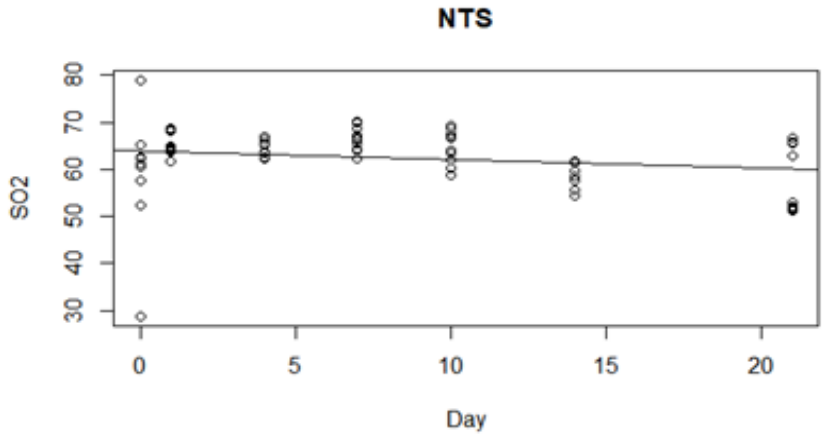

Figure 3. Regression analysis for the NTS data set. Day is displayed on the x axis and SO_2 values are displayed on the y axis. No significant change was found over time. A trendline is shown to see the tendency of the data over time.

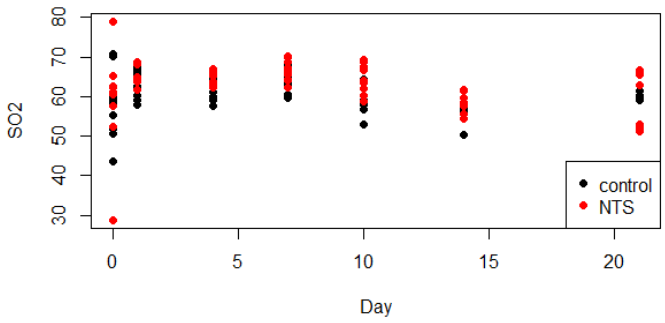

Figure 4. SO_2 values used to find a correlation between day and mouse group. SO_2 values are listed as a percent. No significant difference was found between groups over time.

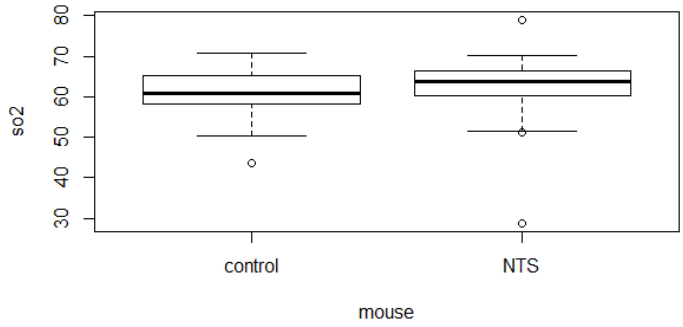

Figure 5. Box plot of SO_2 values used to show comparison of mean values between groups. No significant difference was found between means.

Discussion

Lupus nephritis is present in approximately 60 percent of SLE patients (Lupus Foundation of America 2013). With kidney disease being such a big problem in patients with lupus, it is important to be able to easily detect and check for kidney disease regularly. Previous research shows that 18-20 percent of patients that are re-biopsied progress from a non-proliferative stage to a proliferative stage of renal disease (Narváez et al. 2017). New technology, such as PAI, has also been used in previous research for the detection of cancers (Favazza et. al 2011). The goal of this research was to determine whether technology like this is able to recognize kidney disease in lupus patients. Because PAI is a non-invasive technique, kidney disease could be discovered earlier and checked more often.

The main focus of this study was to determine if photoacoustic technology could detect both disease activity, and disease progression. In order to determine if disease was present in the NTS injected mice, we used SDS-PAGE to check for albumin in their urine. The high presence of albumin allowed us to confirm that the mice had GN (urine tests were sufficient to determine GN because we had control urine samples for each mouse, and the NTS model is notoriously successful in literature). Then using the results from the MSOT, we found that disease activity could not be found, because the p value of the ACOVA was 0.7325 ($p<0.05$ is considered significant) indicating that the control mouse group and the NTS mouse group were not significantly different in SO_2 values. Second, we found that disease progression could also not recognized by the MSOT. We determined this by looking at change in SO_2 value overtime. This test had a p value of 0.110 indicating no significant difference between SO_2 values over 21 days. Therefore, we reject our hypothesis that photoacoustic technology would be able to detect disease activity and progression.

There were several limitations of this study. First, the MSOT technology is very new, and has never been used before in an attempt to detect lupus nephritis (Kim, personal communication June 2019). One of the first major limitations was that it takes nearly an hour to image each mouse. We were also only able to image a maximum of six mice a day due to time limitations which resulted in having a very small sample size by the end of the project. Another limitation was that it is not fully capable of detecting lipids, which we wanted to use as another indicator of disease. Lipid accumulation can lead to injury of organs such as the kidney, so detection of lipids could help determine LN (Bobulescu 2010). Then when performing data analysis, the selection of the region of interest can be difficult when looking at the MSOT images that were reconstructed. It is extremely important to select the area precisely in order to get accurate results, however it can be hard to distinguish between organs and other surrounding tissues which could potentially skew the results (Figure 6).

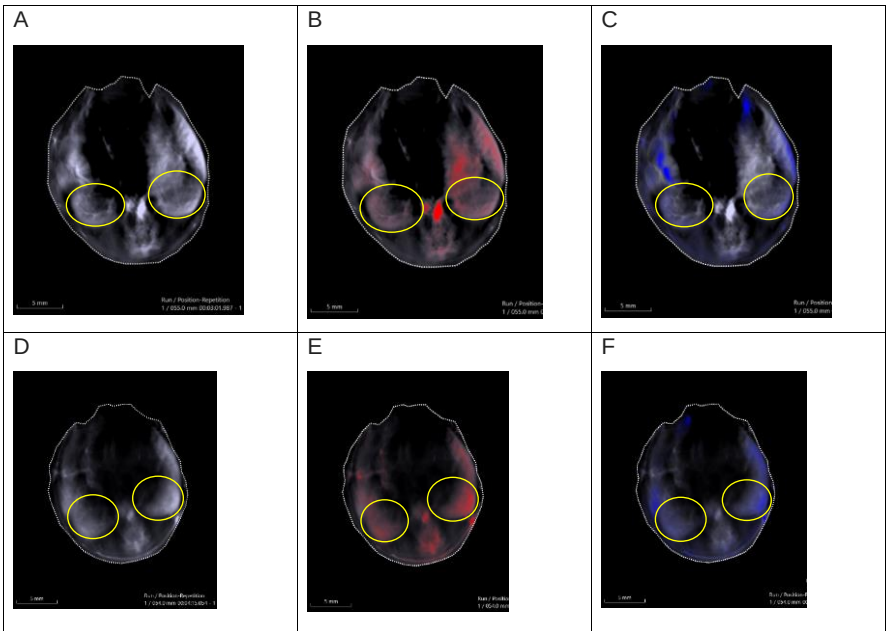

Figure 6. A-C control mouse raw image, oxygenated hemoglobin image, and deoxygenated hemoglobin image respectively. D-F NTS mouse raw image, oxygenated hemoglobin image, and deoxygenated hemoglobin image respectively. The approximation of the kidney regions are circled in yellow.

Lastly, lupus nephritis occurs mainly in the cortex of the kidney, so using the whole volume of the kidney could weaken the results because the SO_2 values of the unaffected medulla are included in the data set. Due to these limitations of the study, there are many improvements that can be made in order to retest our hypothesis about the use of PAI to identify lupus nephritis.

In the future we would like to run several more experiments in order to improve our previous work. Initially, it would be beneficial to be able to detect lipid signals in the kidney which could possibly be done using D_2O instead of deionized water within the imaging chamber. We would also want to try to distinguish the cortex of the kidney from the medulla when selecting a region of interest for analysis. After reviewing some of the images, we found that it may be possible to select only the cortex using cross sections of the kidney. The MSOT technology is still very new, so further improvements with the technology's functionality will also be beneficial to our study.

Furthermore, previous research using photoacoustic technology also came across some of the same problems when using the MSOT. In a study looking at skin lesions, they found it difficult to accurately determine the depth of the lesion because they were unable to determine if the light waves had made it completely through the lesion (Favazza et al. 2011). In another study

testing for lung cancers they also had many similar problems to our study. They found that it was difficult to get a high-resolution image in order to detect where exactly the cancer was located (Guo et. al, 2017). This is similar to our study in that the images were not very clear so it was difficult to select the exact kidney as the region of interest, which could potentially lead to error. Guo et al. (2017) also discussed another problem that our future work will eventually have when we start using human subjects. The MSOT machine has a small imaging chamber that would not be able to hold a human body. Therefore, it would be important to come up with some type of probe, such as the one used for an ultrasound, in order to be able to image a human kidney.

Even though the results of this study were not what we expected, there is still a lot of work to be done in the future that could potentially benefit thousands of lupus patients. Patients would benefit from not only a non-invasive imaging technique, but renal disease could be checked sooner, and more often. Photoacoustic imaging has a lot of potential in the future, as it is already used to detect some cancers and other tumors, such as on the skin and tongue (Favazza et. al 2011 and Guo et. al 2017). Finding a technology that can detect for lupus nephritis early on is crucial to patient's health. A patient that has a positive urine test for albumin, which indicates renal disease, may have already had several inflammatory events in their kidneys (Kim, personal communication June 2019). Renal damage is one of the most serious complications is SLE patients, so it is important to be able to recognize the signs and symptoms earlier than available to doctors now.

Works Cited

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