ARTICLE TYPE

Multiphoton imaging of the effect of monosaccharide diffusion and formation of fluorescent advanced end products in porcine aorta

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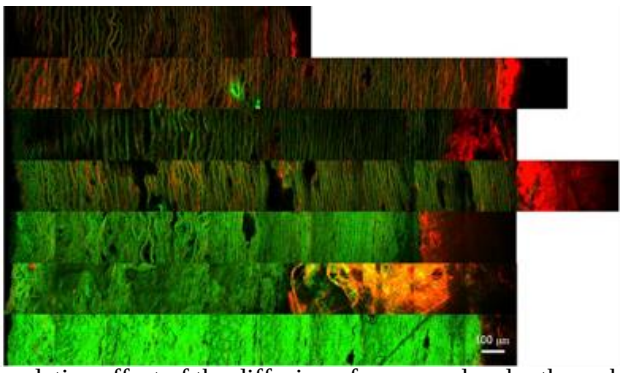


Prolonged exposure of tissues to elevated blood sugar levels lead to the formation of advanced glycation end products (AGEs), thus contributing to diabetic complications. Since the vascular system is in immediate contact with blood, diabetic effects on aorta is a major health concern. However, the relative effect of the diffusion of sugar molecular through the vascular wall and the rate of AGE formation is not known. In this study, we aim to address this issue by incubating excised porcine aorta in D-glucose, D-galactose, and D-fructose solutions for different periods. The tissue specimens were then excised for multiphoton imaging of the autofluorescence intensity profile across the aorta wall. We found that for Days 4 to 48 incubation, autofluorescence is constant along the radial direction of the aorta sections, suggesting that monosaccharide diffusion is rapid in comparison to the rate of formation of fluorescent AGEs (fAGEs). Moreover, we found that in porcine aorta, the rate of fAGE formation of D-fructose and D-glucose are factors 2.08 and 1.14 that of D-galactose. Our results suggest that for prolonged exposure of the cardiovascular system to elevated monosaccharides 4 days or longer, the damage to the aorta is uniform throughout the tissues.

KEYWORDS

multiphoton imaging, diabetes, advanced glycation end products (AGEs), aorta, diffusion, autofluorescence

1 | INTRODUCTION

Diabetes mellitus (DM) a major health problem world-wide. In 2015, it was estimated that 415 million are affected by this disease, corresponding to 8.8% of the global population in the age range of 20-79. By 2040, the projected prevalence will reach 642 million. Since DM is a long-term medical condition, the cost for patient care is staggering.[1] As is well-know, elevated sugar levels lead to non-enzymatic Maillard reaction, leading to the formation of Schiff bases which are later converted to Amadori products. While these are reversible intermediate glycation products, they eventually convert to long-lived and irreversible advanced glycation end products (AGE). While tissue glycation occurs with normal aging, AGE accumulation is accelerated in diabetic patients. AGE affect normal physiological function by the formation of additional cross-links in connective tissues and alteration of cellular function by binding to AGE receptors (RAGE) which can result in the production of reactive oxygen species.[2-4] AGE accumulation in tissues have been implicated in the diabetic complications such as retinopathy, nephropathy, neuropathy, and cardiovascular diseases.[5-8] The roles of AGE in diabetic pathogenesis also led to concerns in diet input of AGE as Maillard reaction is accelerated at higher temperatures and leads to food browning.[9, 10] In fact, feeding laboratory animals

with AGE can lead to atherosclerosis and kidney diseases.[11, 12] AGE inhibitors such as aminoguanadine were shown to be effective in reducing pathogenesis of diabetic complications.[7, 13] Other molecules such as 3-phenyacyl-4,5-dimethylthiazolium chloride is able to improve compliance in stiffened arteries.[14] Due to the importance of AGE inhibition, various strategies, including the use of various plant extracts, have been investigated.[15-17] Among the variety of AGE species, some, such as pentosidine, are known to be fluorescent.[16, 18] While immunoassay is the leading methodology in analyzing tissue's AGE content, they are invasive in nature. Therefore, the development of non-invasive diagnostic tool based on the detection of fluorescent AGE (fAGE) will be invaluable in the clinical setting. Such a technology will also allow the evaluation of the effectiveness of AGE inhibitors as drug molecules. Therefore, in order to develop the optimal technology, there is a need to clarify the effects of dominant dietary sugar molecules in the formation of fAGE in tissues.

A major pathological challenge of diabetic patients is cardiovascular complications. Due to the immediate contact of blood, the vascular system is expected to be most immediately affected by a rise in blood monosaccharide levels. Specifically, it was found that the level of skin collagen autofluorescence increases with the severity of stiffness of the artery and joints.[19] In addition to the fact that glucose, fructose, and galactose are the three primary dietary monosaccharides, studies have also shown that fructose-enriched diet can lead to hypertension and renal microvascular damage in rats.[20, 21] Similarly, galactose-enriched diet can increase vascular permeability and causes retinal microvascular lesions.[22, 23] Since fructose has become a major food additive, there is particular concern of the pathogenic effects of fructose consumption.[24]

Furthermore, since aorta is on the order of a few millimeters in thickness, one expect there to be a gradient of diabetic pathogenesis with a decrease in severity away from the vascular center. Therefore, understanding how monosaccharide diffusion across the vessel wall and the rate of AGE formation can lead to improved understanding of diabetic pathogenesis in the cardiovascular system. Furthermore, since the rate of glycation is different for different monosaccharides, with aldoses, in general, being more reactive than ketoses, there is a need to investigate how different monosaccharides affect cardiovascular pathogenesis. For commonly simple sugars found in human diets, sugar reactivity with the amino group in forming Schiff's base is found to be in the order of glucose, galactose, and fructose.[25]

In this study, we study the interplay between diffusion and fAGE formation in porcine aorta. Since disaccharides and polysaccharides such as sucrose, lactose, and starch require enzymatic digestion into monosaccharides before intestinal absorption[26] we will focus on monosaccharides in this study. By exposing the inner wall of the aorta to the same concentration of D-glucose, D-fructose, and D-galactose, we propose to study the spatial and temporal profiles of fAGE formation.

2 | MATERIALS AND METHODS

2.1 | Porcine aorta glycation

Porcine aorta was acquired from a local market. Two sections of the same vessel were used. The two sections are respectively, 3-6 cm and 7-10 cm from the heart. After washing three times with PBS, the aorta sections were sterilized in 4% iodine solution for 1 minute. After sterilization, the aorta sections were again washed three times with PBS and exterior of the aorta specimens were dried with KimwipesTM (Kimberly-Clark, Roswell, GA) that were treated under UV illumination inside a biosafety cabinet 30 minutes. To prevent exposure to the monosaccharide solutions, the outer surface and cross sections at the two ends of the aorta were evenly sprayed with polytetrafluoroethelyne (PTFE) coating (Cogelsa, Spain). The tubular aorta sections were then separately immersed in three monosaccharide solutions. The three monosaccharides we used were D-glucose (Sigma-Aldrich, St. Louis, MO), D-fructose (Sigma-Aldrich, St. Louis, MO), and D-galactose (Acros Organics, Fair Lawn, NJ). In each case, the aorta was treated in 0.5 M sugar (5×PBS) composed of 1% Penicillin-Streptomycin (10,000 U/mL, Gibco, Waltham, MA). Each aorta section was placed in a 50 ml conical tube and immersed with 30 ml of the monosaccharide solution. The incubation was performed inside an incubator at 37 ℃. Every four days, the monosaccharide solution was replaced. Furthermore, every twelve days, the exterior of aorta specimens were dried and re-sprayed with polytetrafluoroethelyne (PTFE). At selected time points, the aorta sections were removed, frozen for 30 minutes and a middle section 3 mm in length was excised with a razor blade. We performed experiments at 8 different time points: Days 0, 4, 8, 12 24, 36, and 48. The aorta section was then placed on a glass-bottom Petri dish for multiphoton imaging. To acquire statistically significant data, at each time point, two aorta sections were treated with each monosaccharide solution. Continuous multiphoton images of adjacent positions were acquired, thus achieving an autofluorescence and SHG map of the specimen from the interior to exterior regions of the aorta. Three maps of each aorta sections were acquired. Therefore, six interior-to-exterior SHG and AF maps were acquired at each time point.

2.2 | Multiphoton microscope

Multiphoton imaging was performed on a home-built system based on an inverted microscope (TE2000U, Nikon, Japan). The excitation source was a titanium-sapphire (ti-sa) laser (Tsunami®, Spectra Physics, Santa Clara, CA) pumped by a diode-pumped, solid-state laser operating at 532 nm (Millennia® Pro, Spectra Physics). 780 nm output of the ti-sa laser was used as the excitation source. Upon reflection from a galvanometer-driven, x-y scanning system (6215M, Cambridge Technology, Watertown, MA), the laser was beam-expanded and reflected into the focusing objective (S Fluor, 20×/NA 0.75, Nikon) by a primary dichroic mirror (720dcspxr, Chroma Technology, Bellows Falls, VT). On sample power was approximately 80 mW. Second harmonic generation (SHG) and specimen autofluorescence was collected using the objective in the epi-illuminated geometry. After short-pass filtering by the primary dichroic and further separated by additional dichroic mirrors (420dclr, 495dcxr, and 550 dcxr, Semrock, Rochester, NY) and filtered by additional bandpass filters (FF01-390/18, Semrock; HQ460/50, Chroma

Technology; FF03-525/50, FF01-630/92, Semrock), separate detection of SHG (390 ± 9 nm), blue (460 ± 50 nm), green (525 ± 50 nm), and red (630 ± 92 nm) fluorescence was achieved. Single photon counting photomultipliers (R7400P, Hamamatsu, Hamamatsu City, Japan) were used to detect signal photons. After the acquisition of teach optical scan (area of 235×235 μm^2), the specimen is translated by a mechanical stage (Prior Scientific, UK) to achieve large-area, cross-sectional scan of the aorta. Signal background was determined by averaging 2 regions each approximately 400 pixels in the glass slide where no tissue is present..

3 | RESULTS AND DISCUSSION

Shown in Fig. 1-3 are representative interior-to-exterior AF and SHG map for D-glucose, D-galactose, and D-fructose. Qualitatively, AF level increased with increased incubation time. Furthermore, at the longer incubation time points (Days 24, 36, and 48), there is a clear increase in AF from D-glucose, D-galactose, and D-fructose treated specimens.

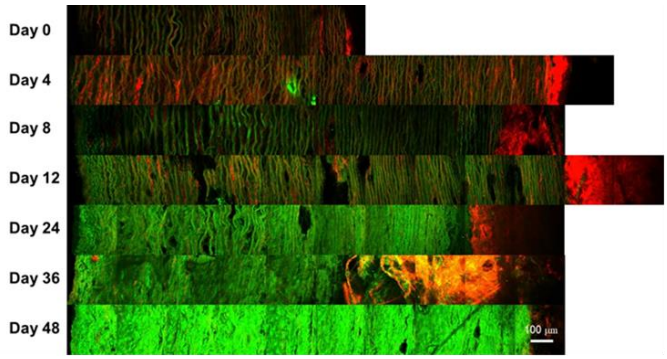


FIGURE 1 Representative multiphoton autofluorescence (AF) and second harmonic generation (SHG) imaging of D-fructose treated porcine aorta section from interior (left) to exterior (right) at Days 0, 4, 8, 12 24, 26, and 48.

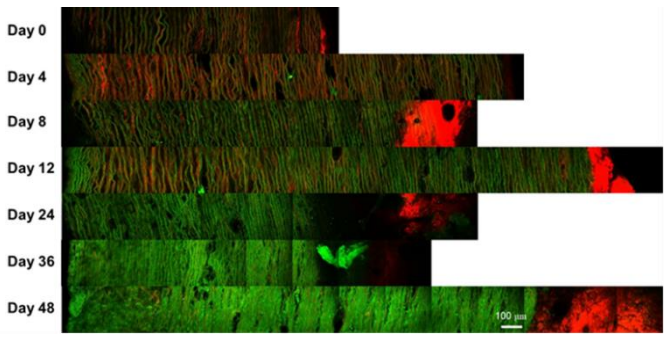
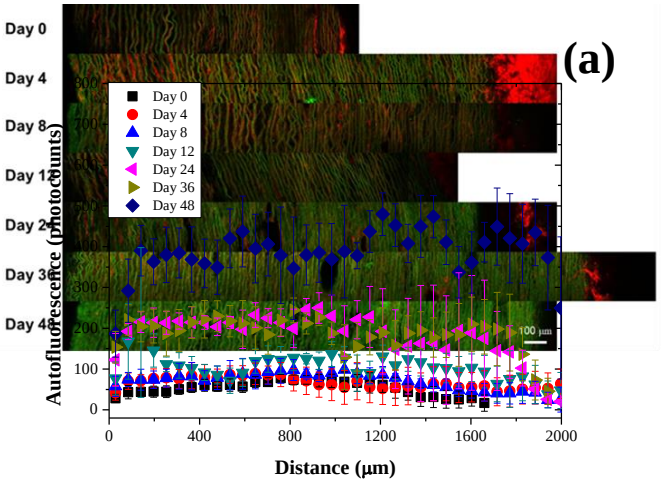


FIGURE 2 Representative multiphoton autofluorescence (AF) and second harmonic generation (SHG) imaging of D-galactose treated porcine aorta section from interior (left) to exterior (right) at Days 0, 4, 8, 12 24, 36, and 48.

FIGURE 3 Representative multiphoton autofluorescence (AF) and second harmonic generation (SHG) imaging of D-glucose treated porcine aorta section from interior (left) to exterior (right) at Days 0, 4, 8, 12 24, 36, and 48.

To provide a quantitative evaluation of the spatial AF profiles, AF signal within a 60 μm horizontal strip was computed, and the results for the three monosaccharides specimens are plotted in Fig. 4. As the results show, regardless of the incubation time, tissue autofluorescence is steady as a function of depth for all three monosaccharides.



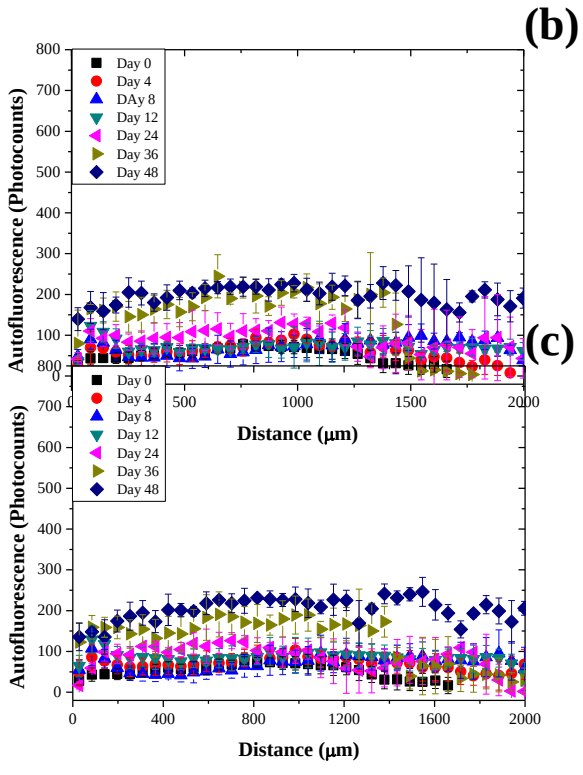


FIGURE 4 Multiphoton autofluorescence (AF) profiles for porcine aorta specimens treated with (a). D-fructose, (b). D-galactose, and (c). D-glucose. AF intensity was obtained by summing AF within 60 μm wide strips**.**

To further quantify the rates of rates of AF increase, the time-dependent AF profiles in Fig. 4 were fitted to a linear model and the y-intercept representing the average AF across the aorta cross section at each time point was plotted in Fig. 5. The slopes, characterizing the rate of AF increase with time, were found to be 5.65±0.57, 2.72±0.64, and 3.09±0.62 (photon counts/day) for D-fructose, D-galactose, and D-glucose, respectively. Therefore, in porcine aorta, D-fructose was most effective in inducing fluorescent byproducts from tissue glycation, with similar efficiency for D-galactose and D-glucose.

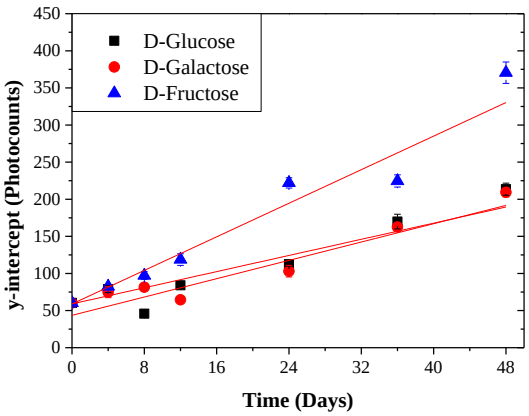


FIGURE 5 Rate of formation of D-glucose, D-galactose, and D-fructose induced tissue autofluorescence in porcine aorta.

We used porcine aorta as a proof of principle study demonstrating the effects of the diffusion of different monosaccharides in fAGE formation. Our results conclusively demonstrated that autofluorescence increase may be used as a biomarker for tissue glycation. We plan to extend this study by designing multiphoton endoscopic probe that can be inserted into the vasculature *in vivo* and for fAGE measurements. This will be demonstrated on diabetic murine models first before being applied to humans. Specifically, rats will undergo diabetic induction using streptozotocin (STZ). Diabetic rats will then be fed diets containing the three monosaccharides used in this study (D-glucose, D-galactose, and D-fructose). The multiphoton endoscopic probe will be used to study fAGE formation in the animals at different time points. For validation of the in vivo results, we also plan to perform *ex vivo* studies in which the animals will be sacrificed, and aorta removed for fAGE analysis. In this case, we will perform the comparison on multiple animals at each time point to validate our results. A number of non-diabetic animals will also be used as controls. The use of multiple animals would validate our results statistically. If successful, our approach would allow the direct monitoring of diabetic complications of the cardiovascular system in human.

4 | CONCLUSION

In this study, we simulated the effect of elevated monosaccharide in the formation of advanced glycation end products adducts in porcine aorta. By only exposing the aorta interior to the three monosaccharides commonly found in human diets (D-glucose, D-galactose, and D-fructose), we excised and imaged the aorta cross-sections for up to 48 days of incubation. We found that for Days 4 to 48 incubation, autofluorescence is constant along the radial direction of the aorta sections, suggesting that monosaccharide diffusion is rapid in comparison to the rate of formation of fluorescent AGEs (fAGEs). Moreover, we found that in porcine aorta, the rate of fAGE formation of D-fructose and D-glucose are factors 2.08 and 1.14 that of D-galactose. Our results suggest that for prolonged exposure of the cardiovascular system to elevated monosaccharides 4 days or longer, the damage to the aorta is uniform throughout the tissues.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Science and Technology, Taiwan, R. O. C. (MOST 107-2112-M-002 -023 -MY3)

AUTHOR CONTRIBUTIONS

Chih-Ju Lin participated in experiment design and data analysis. Sheng-Lin. Lee helped with imaging program. Jeon-Woong Kang and Peter T.C. So participated in the discussion of this research. Chen-Yuan Dong edited the manuscript.

FINANCIAL DISCLOSURE

The authors declare no conflicts of interest.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings in the manuscript will be available upon request to the corresponding authors.

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Chih-Ju Lin received his PhD degree in Physics from National Taiwan University, and he received his master degree from National Changhwa University of Education. His research interests are focusing on multiphoton imaging of hepatobiliary metabolism *in vivo* and the autofluorescence of glycation tissues. He also has excellent skills to perform animal experiments.

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