Medical Research Archives

European Society of Medicine

OPEN ACCESS

Published: February 29, 2024

Citation: Miura K and Iwashita T, 2024. Histological Visualization of Glyceraldehyde-Derived Glycation with Glucose using Ultrasound Microscopy, Medical Research Archives, [online] 12(2). https://doi.org/10.18103/mra.v12i2.5035

DOI
https://doi.org/10.18103/mra.v12i2.5035

ISSN: 2375-1924

RESEARCH ARTICLE

Histological Visualization of Glyceraldehyde-Derived Glycation with Glucose using Ultrasound Microscopy

Katsutoshi Miura*[1], Toshihide Iwashita[1]

[1] Department of Regenerative & Infectious Pathology, Hamamatsu University School of Medicine, Japan

***Corresponding author:** kmiura.hama.med@gmail.com

ABSTRACT

Background: Reducing sugars and reactive aldehydes, such as glyceraldehyde (GA), non-enzymatically react with proteins to form advanced glycation end-products (AGEs). GA is produced in the glycolysis pathway, and GA-derived AGEs play an essential role in the pathogenesis of angiopathy associated with hyperglycemia in patients with diabetes.

Aims: Several studies have reported on chemical alterations in glycation. However, histological confirmation of these biochemical changes has been relatively rare. This study aimed to visualize glyceraldehyde-induced glycation and evaluate the severity of glycation using attenuation of sound (AOS) values. Given that glycation promotes cross-links between proteins and sugars, the energy loss of sound that passes through them increases. We hypothesized that AOS alteration would reflect the glycation state of the tissues and cells.

Methods: Fresh frozen sections or fresh cells were briefly fixed in ethanol and soaked in GA with different glucose concentrations. Thereafter, AOS images were obtained via scanning acoustic microscopy over time, and tissue and cellular glycation induced by GA with glucose was evaluated using AOS values.

Results: AOS images were able to visualize GA-induced glycation over time. Compared to GA alone, glucose supplements concentration-dependently accelerated glycation. The arterial smooth muscle, collagen, and intima were apt to accept glycation, whereas the mucosa was unaffected.

Conclusion: The comparability and digital nature of AOS images make them suitable for statistical analysis of glycation. Higher glucose concentrations promoted a greater increase in the AOS values of the sections and cells. Moreover, the increase in AOS values varied according to organs and cells, which supports the difference in affected organs among patients with diabetes mellitus. Our findings suggest that a longer hyperglycemic state promotes greater glycation.

Keywords: ultrasound, glycation, advanced glycation end-products, glyceraldehyde, diabetes mellitus, attenuation of sound, microscopy

Introduction

Reducing sugars and reactive aldehydes, such as glyceraldehyde, non-enzymatically react with amino or guanidino groups of proteins to form advanced glycation end-products (AGEs) via the Maillard reaction, which involves Schiff base formation followed by Amadori rearrangement.[1] Glyceraldehyde is produced through the glycolysis pathway that converts glucose into pyruvate[2] but can also be created in vivo, most likely through the aldolase-catalyzed conversion of fructose 1-phosphate during polyol metabolism, which is expedited under high glucose conditions.[3] Short-chain aldehydes, including glyceraldehyde, react with amino and thiol groups of the proteins to reduce the protein's function.[4]

Glyceraldehyde-derived AGEs (Glycer-AGEs) have been reported to be produced through the Maillard reaction in which Nα-acetyl-lysine and Nα-acetyl-arginine react with glyceraldehyde under physiological conditions.[5] Glycer-AGEs play an essential role in the pathogenesis of angiopathy in diabetes, aging, and neurodegenerative diseases associated with hyperglycemia.[6]

Several studies have investigated structural alterations in glycation. However, histological confirmation of these biochemical changes has been relatively rare. This could be attributed to the need for unique techniques, such as staining, to obtain histological data and subjective and analog results that make objective evaluation difficult.

Periodic acid-Schiff (PAS) staining has been used to detect structures containing a high proportion of carbohydrate macromolecules, such as glycogen, glycoprotein, and proteoglycans.[7] Substances with nearby glycol groups or amino or alkylamino derivatives are oxidized by periodic acid to form dialdehydes, which combine with the Schiff reagent to form an insoluble magenta compound. Diseases associated with diabetes, such as Kimmelstiel–Wilson nodules in the glomerulus[8], show positive results during PAS staining. However, predicting the severity of diabetes-associated diseases remains difficult.

Scanning acoustic microscopy (SAM) detects the attenuation of sound (AOS) through sections or cells.[9] Attenuation is the loss of intensity and amplitude as sound waves travel through a medium. Absorption, which involves converting acoustic energy into heat, is the primary source of attenuation in soft tissue. Additionally, scatter is known to contribute to AOS. Short-wavelength ultrasound waves are necessary for higher resolution during histology. However, short-wavelength frequencies indicate high-frequency waves, which lose more energy than low-frequency waves. The extent of attenuation depends on the tissue type through which the sound wave travels. The intrinsic propensity of attenuation at a given frequency is represented by its attenuation coefficient (α) and measured in dB/MHz × cm. Accordingly, research has shown that the bone, kidneys, fat, and blood have an α of 20, 1, 0.6, and 0.18 dB/cm at 1 MHz, respectively,[10] indicating that the AOS increases with the density and stiffness of the tissue.[11] We, therefore, hypothesized that glycation would increase the AOS values based on the degree of cross-linking.

The current study aimed to visualize glyceraldehyde-induced glycation and evaluate the severity of glycation using AOS values. Given that glycation promotes cross-links between proteins and sugars, the energy loss of sound that passes through them increases. We hypothesized that AOS alterations would correspond to the degree of glycation. To test this, we compared AOS images with the corresponding PAS-stained images indicating sugar integration.

Materials and Methods

SAMPLE PREPARATION

Pathology samples not linked to the patient's identity and fresh mouse samples were used for this study. The study protocol conformed with the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of the Hamamatsu University School of Medicine (approval no. 19-180). Written informed consent was obtained from all subjects. All procedures were conducted according to the guidelines and regulations of the ethics committee. For mouse samples, tissues were generously provided by Dr. Y. Enomoto from Regenerative & Infectious Pathology, Hamamatsu University School of Medicine. For fresh frozen sections, tissues were cut into small pieces, frozen at −80°C, and cut into 10-μm-thick sections. For cytology samples, liquid samples were spun down to make single-layer samples. After a definite pathological diagnosis, the remainder of the tissues and cells were used for the SAM study.

TISSUE TREATMENT FOR GLYCATION

Fresh tissue and cell samples were fixed in 95% ethanol for 10–15 min and soaked in phosphate-buffered saline (PBS). The sections were then incubated in the experimental solutions at 37 °C, after which they were removed from the solution, washed in distilled water, and observed via SAM. After SAM observation, the sample was washed in

distilled water and incubated in the same solution over time.

MATERIALS FOR GLYCATION

DL-glyceraldehyde (GA) and glucose were dissolved in PBS to produce a 500 mM GA solution, GA + 50 mM glucose solution, GA + 100 mM glucose solution, and GA + 200 mM glucose solution. Mouse artery and skin were soaked in GA for up to 4 days for GA reaction with tissues. During the incubation, AOS images were observed over time. For supplementary effects of glucose, GA with different glucose concentrations was compared to incubated tissues and cells.

SCANNING ACOUSTIC MICROSCOPY (SAM) OBSERVATIONS

Sections were examined using a SAM system (AMS-50AI; Honda Electronics, Toyohashi, Aichi, Japan) with a central frequency of 320 MHz and a lateral resolution of 3.8 μm,[12,13] The transducer was excited with a 2-ns electrical pulse to emit an acoustic pulse.[14] The section was placed on the stage, and distilled water was used as coupling fluid between the transducer and the section. The transducer was used for transmitting and receiving the wave signal. Waveforms reflected from the surface and the bottom of the sample were compared to measure the AOS at each point. The waveform from a glass surface without the specimen was considered the zero AOS area (black) and used as a reference. The section was scanned along the X–Y axis within 2.4 mm × 2.4 mm, 1.2 mm × 1.2 mm, 0.6 mm × 0.6 mm, and 0.3 mm × 0.3 mm square areas. A single scan required a few minutes. After the scan, the AOS value at each point was plotted on the screen according to color code to generate an AOS image.

LIGHT MICROSCOPIC (LM) OBSERVATION

Before and after final incubation, the slides were used for PAS staining of the tissue and cytology samples to identify glycation areas. LM images were compared with the corresponding AOS images.

STATISTICAL ANALYSES

The mean and standard deviation for the AOS values were calculated from at least five areas per slide. Mean AOS values among different time points after incubation were compared using Student's t-test. A commercial statistics software (BellCurve for Excel; Social Survey Research Information, Tokyo, Japan) was used to calculate the mean areas-of-interest values, generate dot blot graphs, and analyze t-tests. Before statistical analyses, all data sets with normal distribution were compared in a test for the difference between mean values. A p-value of <0.05 indicated statistical significance.

Results

GLYCERALDEHYDE REACTION

Fresh tissues (Fig. 1A: mouse artery, Fig. 1B: mouse skin) were incubated in 500 mM GA over time. AOS values gradually increased after incubation (Fig. 1C, D), with a significant difference being observed between 0 h and day 1 or 2. The corresponding PAS staining displayed little increase in red color intensity. An increase in the AOS values was observed for arterial smooth muscles and perivascular collagen fibers (Fig. 1A) and dermis and epidermis (Fig. 1B).

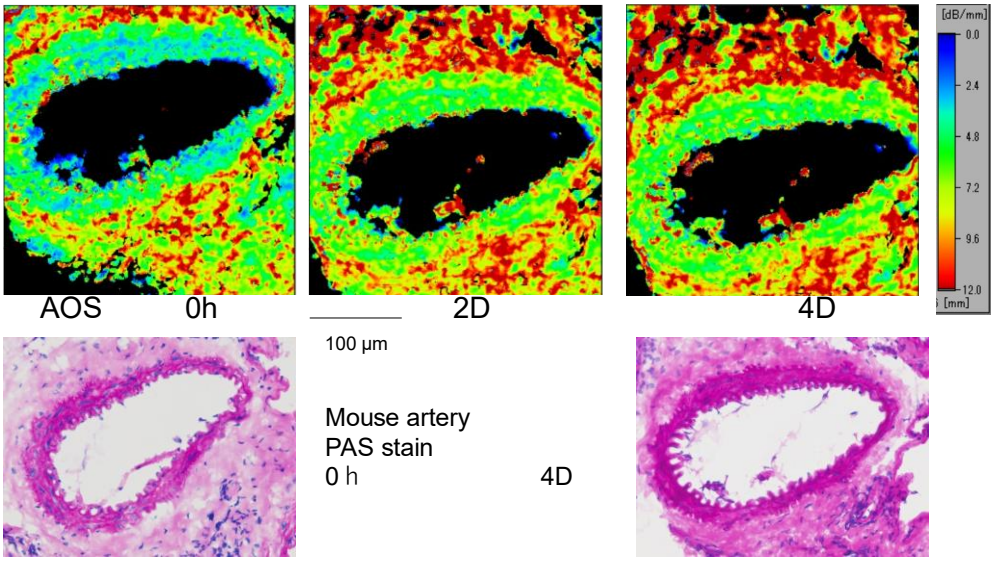


Fig. 1A
Fresh mouse artery incubated in glyceraldehyde. A fresh mouse artery was fixed in ethanol for 15 min, washed in distilled water, and incubated in 500 mM GA over time at 37 °C. The AOS values of the vascular wall and perivascular connective tissues gradually increased after incubation. PAS staining showed that the vascular walls slightly increased positivity after incubation. Upper row: AOS images after incubation at 0 h, 2 days, and 4 days. Lower row: PAS staining at 0 h and 4 days.

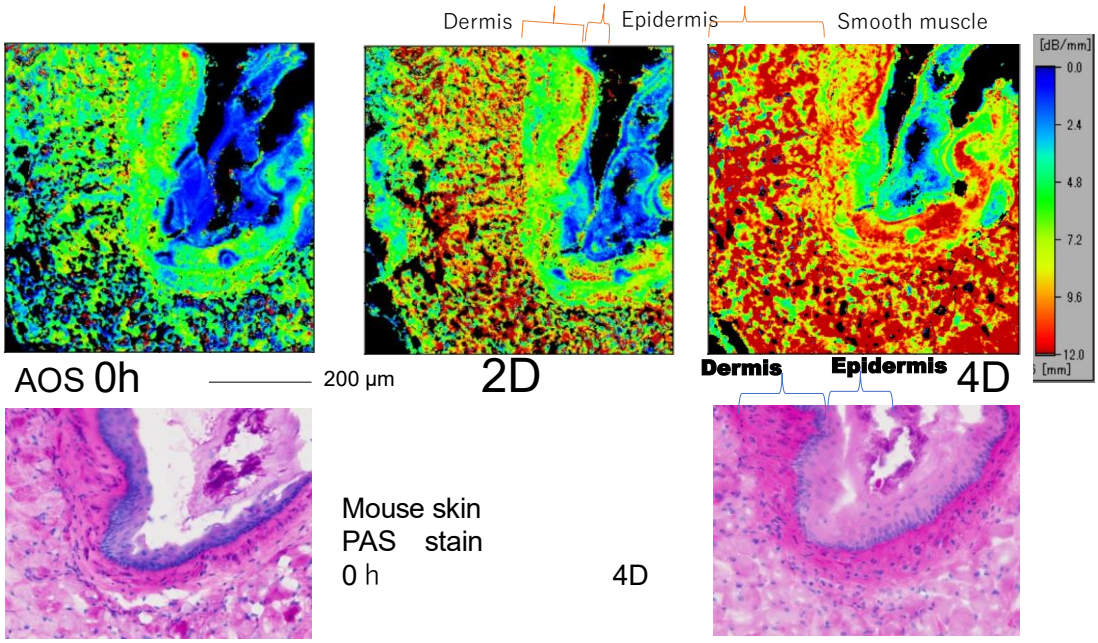


Fig. 1B

Fresh mouse skin after incubation in glyceraldehyde.

The skin epidermis and dermis showed increased AOS values over time. PAS staining showed a slight raised redness after incubation. Upper row: AOS images at 0 h, 2, and 4 days. Lower row: LM images after PAS staining at 0 h and 4 days.

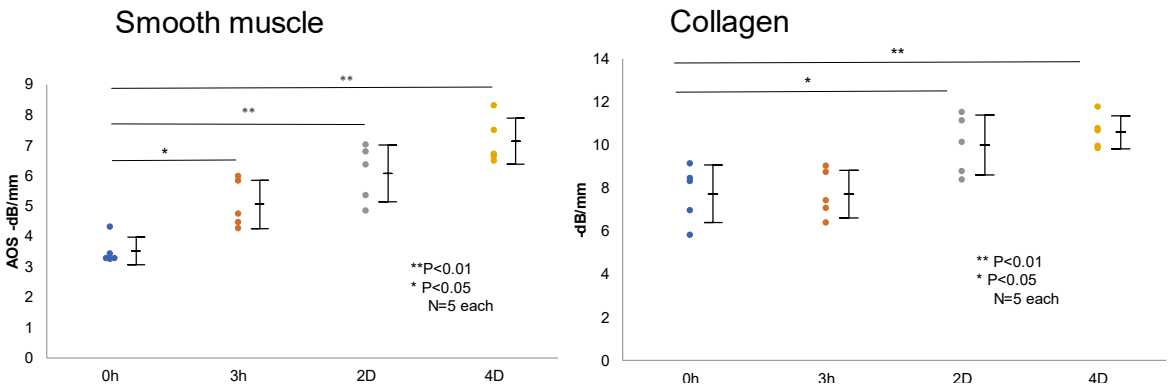


Fig. 1C

Dot blot of AOS values of mouse artery after incubation in glyceraldehyde.

Mean AOS values (+standard deviation) were plotted over time. Both smooth muscles and collagen showed a significant increase in AOS values over time.

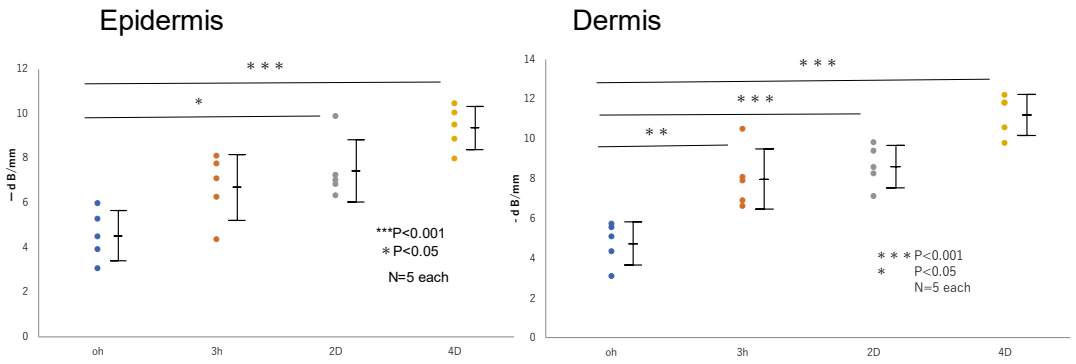


Fig. 1D
Dot blot of mouse skin AOS values (mean + standard deviation) after incubation in glyceraldehyde. The epidermis and dermis showed a significant in increase in AOS values at 2 and 4 days, respectively.

GLUCOSE EFFECTS ON GLYCERALDEHYDE (GA)
The addition of glucose to the GA solution increased the AOS values of the human ureter (Fig. 2A). Ureteral smooth muscles showed a significant increase in AOS values with GA containing glucose than with glucose only at days 1 and 2 (Fig. 2B). The AOS of only GA solution was dramatically greater at day 2 than at 0 h. However, the values were consistently lower than those of GA-containing glucose. PAS staining images showed a higher red color intensity in the GA and GA-containing glucose solution than in the glucose-only PBS solution. The GA + glucose image showed a higher red color intensity than did the GA-only image.

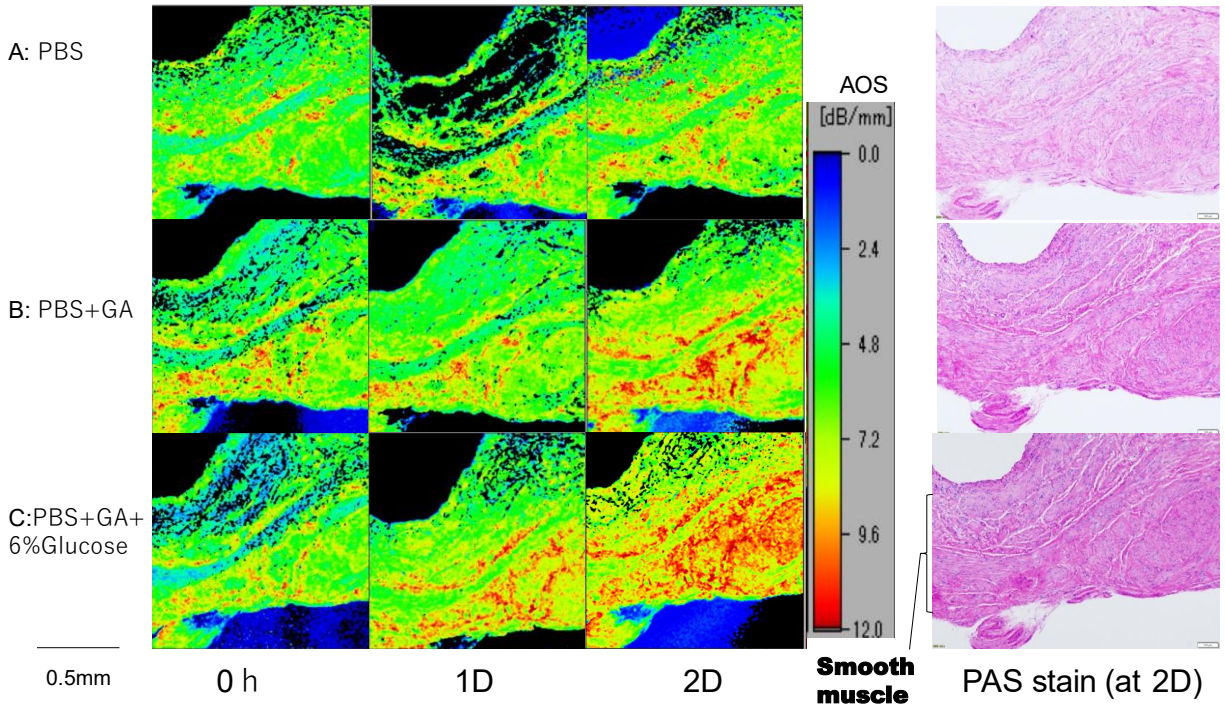


Fig. 2A
Attenuation of sound (AOS) and LM (PAS staining) images of the ureter after incubation with different solutions. The following three groups were compared: A, 200 mM glucose in PBS; B, 500 mM glyceraldehyde (GA) in PBS; C, 500 mM GA and 200 mM glucose in PBS. Smooth muscle AOS values increased over time according to PAS staining intensity.

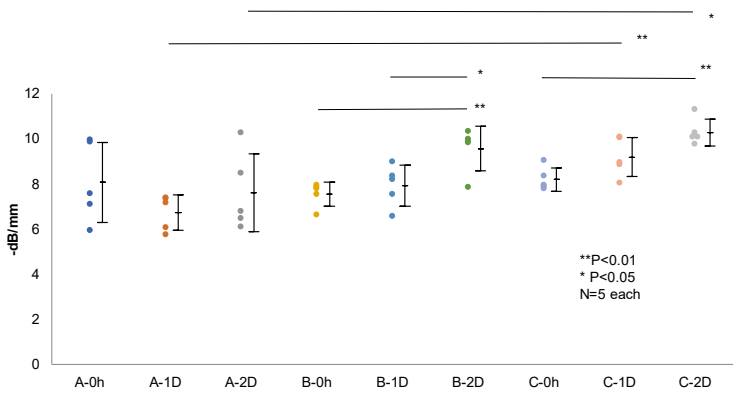


Fig. 2B
Dot blot of AOS values (mean + standard deviation) for the ureter smooth muscle after incubation in the three solutions. Group C showed significantly greater AOS values than did Group A over time. A, 200 mM glucose in PBS; B, 500 mM glyceraldehyde (GA) in PBS; C, 500 mM GA and 200 mM glucose in PBS

EFFECTS OF GLUCOSE CONCENTRATION IN GLYCERALDEHYDE (GA)

We compared AOS values for fresh arteries (Fig. 3A) incubated with different glucose concentrations in GA. Notably, AOS values increased with glucose concentration. However, no remarkable differences in PAS staining results were found between the three concentrations. A noteworthy elevation in AOS values was observed in the smooth muscle of the tunica media. Most AOS values decreased on day 1 after incubation and then increased on day 2. An increase in the AOS was also observed in the tunica intima at day 2 after incubation. Figure 3B shows the results of our statistical analysis for the three glucose concentrations. Compared to smooth muscles incubated without glucose, those incubated with 100 mM glucose showed the highest AOS values, followed by those incubated with 50 mM glucose on days 1 and 2. For the tunica intima, the AOS values in all groups decreased on day 1 and increased on day 2.

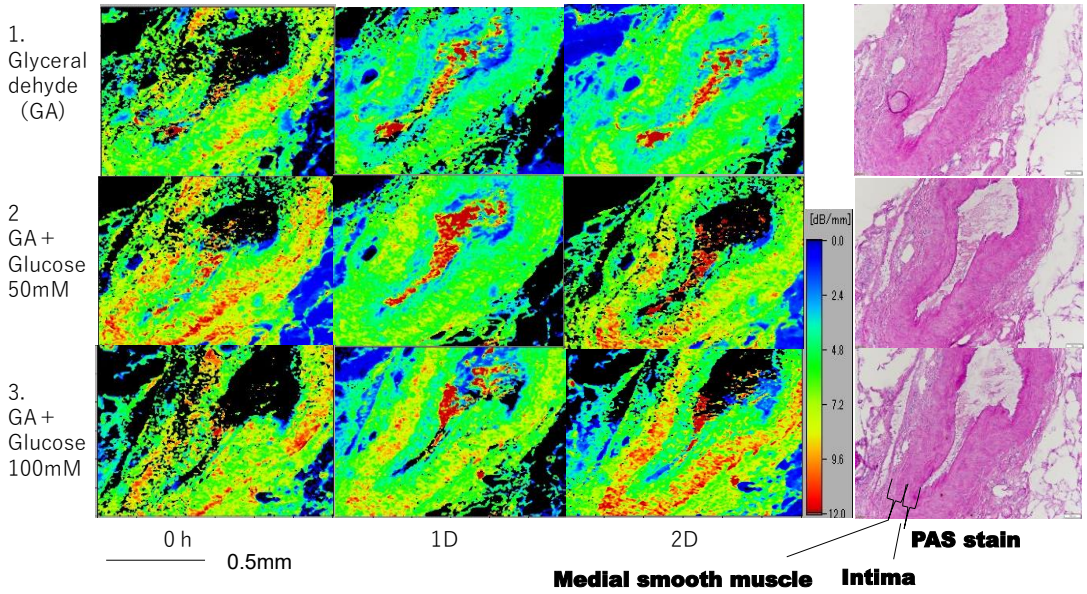


Fig. 3A
AOS and the corresponding PAS staining images for the arteriole under different conditions. Tunica media and intima incubated with 100 mM glucose showed the highest AOS value, followed by those incubated with 50 and 0 mM glucose in decreasing order. PAS staining images showed no remarkable differences. 1: Glyceraldehyde (GA), 2: GA+50 mM glucose. 3: GA+100 mM glucose.

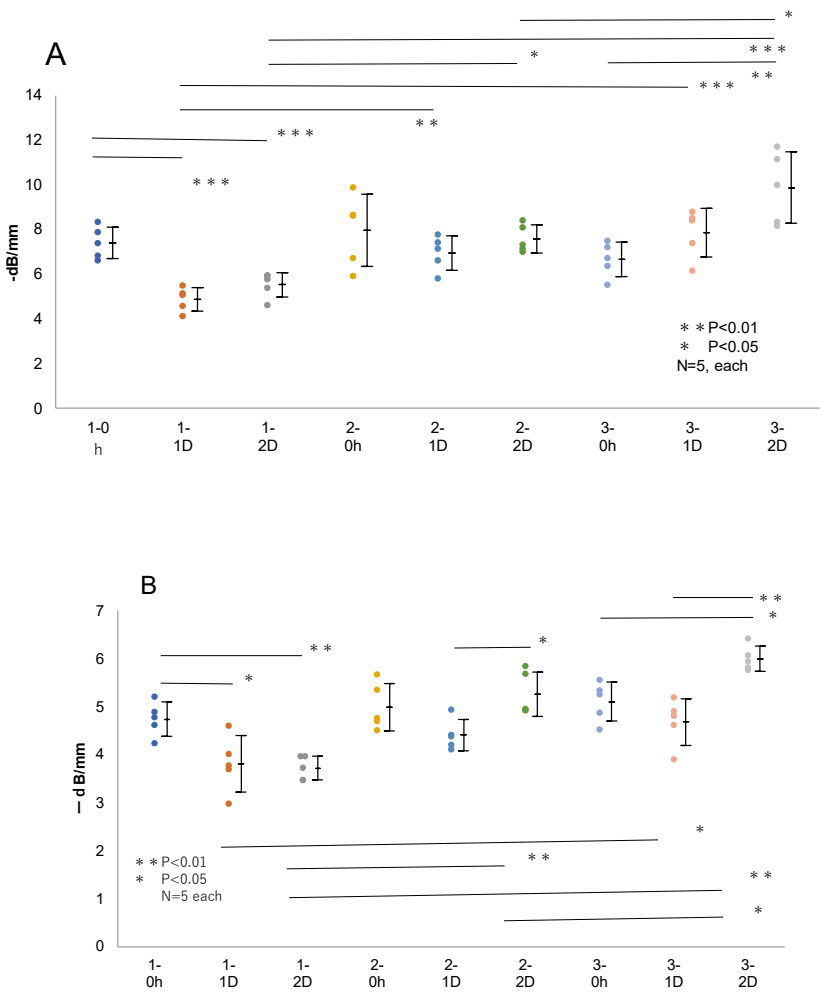
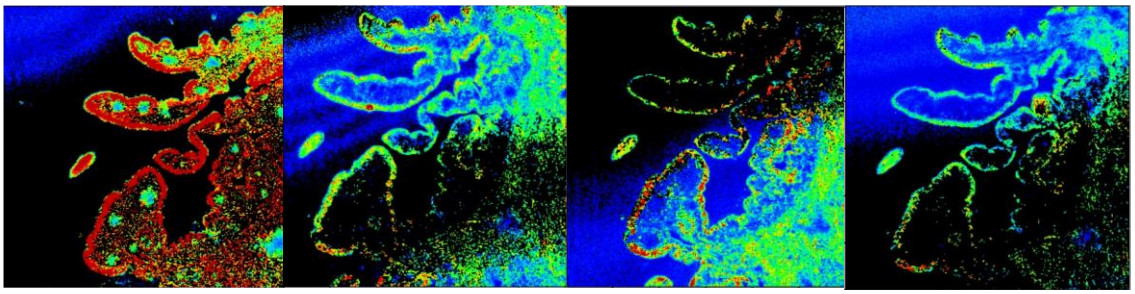


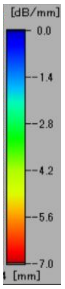
Fig. 3B

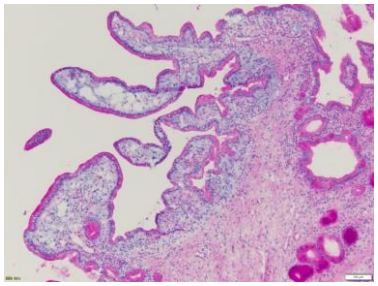
Dot blot of the AOS values for the medial smooth muscle (A) and the tunica intima (B) of arteriole (mean + standard deviation) incubated with three solutions. A: No difference in AOS values was observed between the three groups before incubation. After incubation, the AOS values of Groups 2 and 3 were significantly greater than those for Group 1. Group 3 showed higher AOS than Group 2 at day 2 after incubation. B: No difference in AOS values was observed between the three groups before incubation. After incubation, the AOS values of Groups 2 and 3 were always greater than those for Group 1. At day one, all groups reduced AOS values and Groups 1 and 2 significantly increased at day 2. Comparing the three groups, Group 3 was always the highest after incubation.

Attenuation of sound (AOS) images of the fresh bile duct were compared under different conditions (1, Glyceraldehyde (GA); 2, GA + 50 mM glucose; 3, GA + 100 mM glucose; 4, GA + 200 mM glucose) (Fig. 4). In all groups, the AOS images at 0 h after incubation showed the greatest AOS values. No significant difference was observed between the four groups at any time. Mucosal stromal cells showed a gradual decrease in AOS values after incubation. Mucosal surface and submucosal loose connective tissue consisting of collagen and smooth muscle fibers maintained their AOS values even after 3 days. Small tubules containing PAS-positive mucin revealed no conspicuous images after incubation. The corresponding PAS staining images on day 3 after incubation revealed no remarkable difference among all groups.

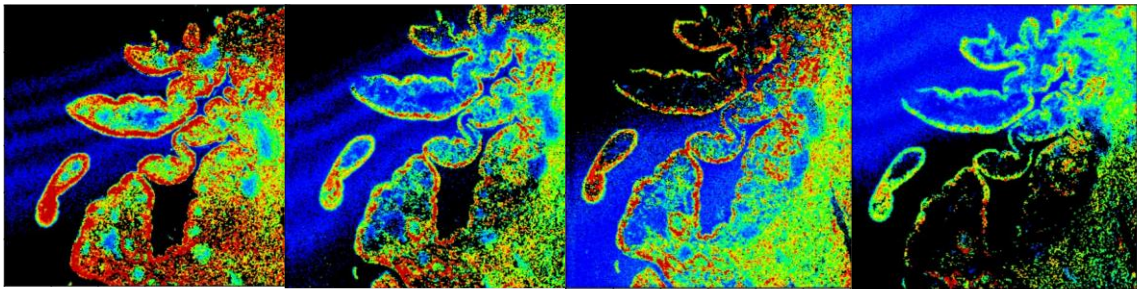


0.5mm

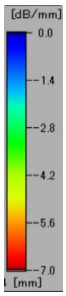


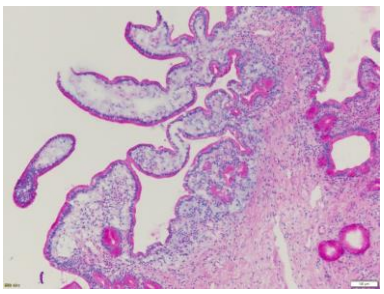


1.Glyceraldehyde (GA)
AOS 0H-1D-2D-3D
PAS stain

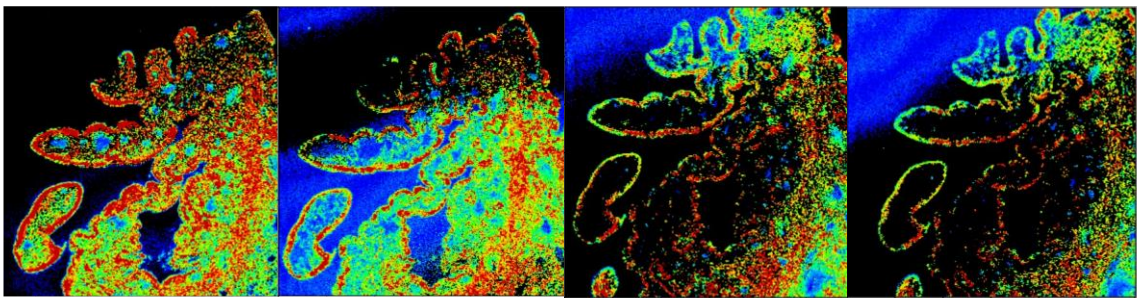


0.5mm

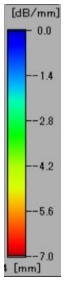


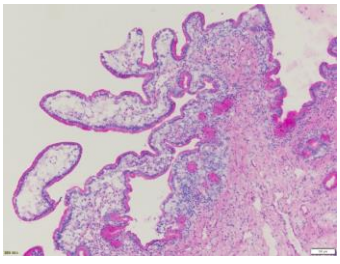


2. GA + Glucose 50mM
AOS 0H-1D-2D-3D
PAS stain



0.5mm





3. GA + Glucose 100 mM
AOS 0H-1D-2D-3D
PAS stain

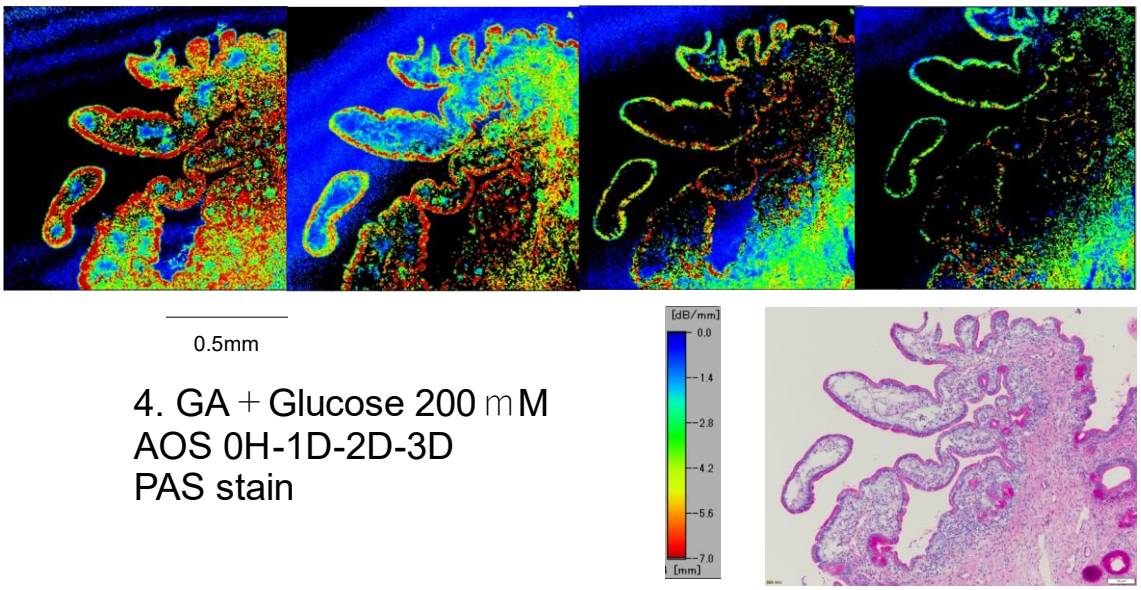
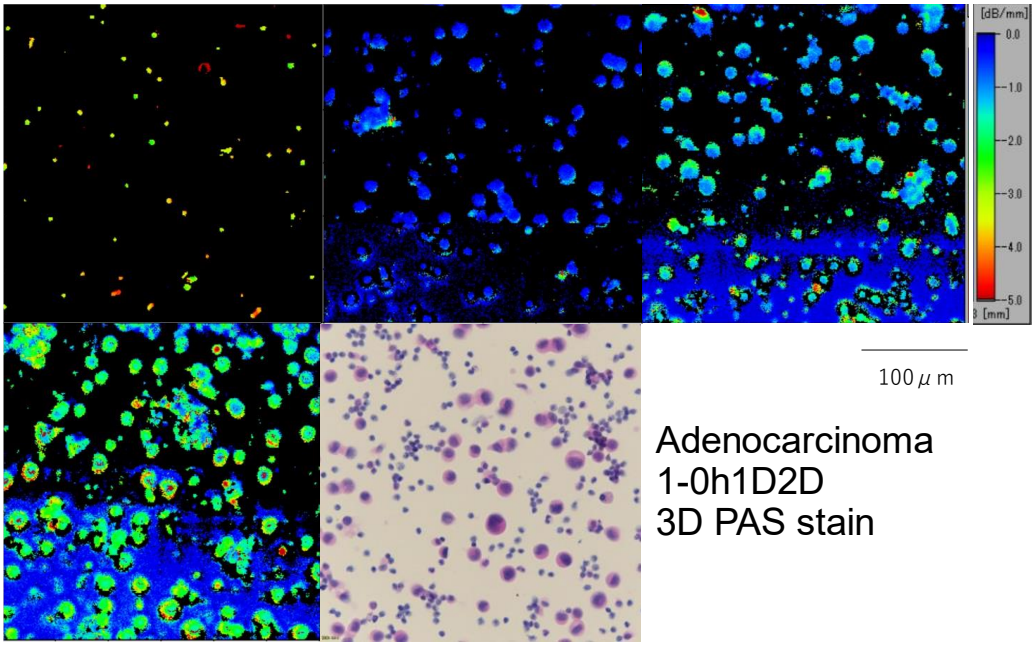
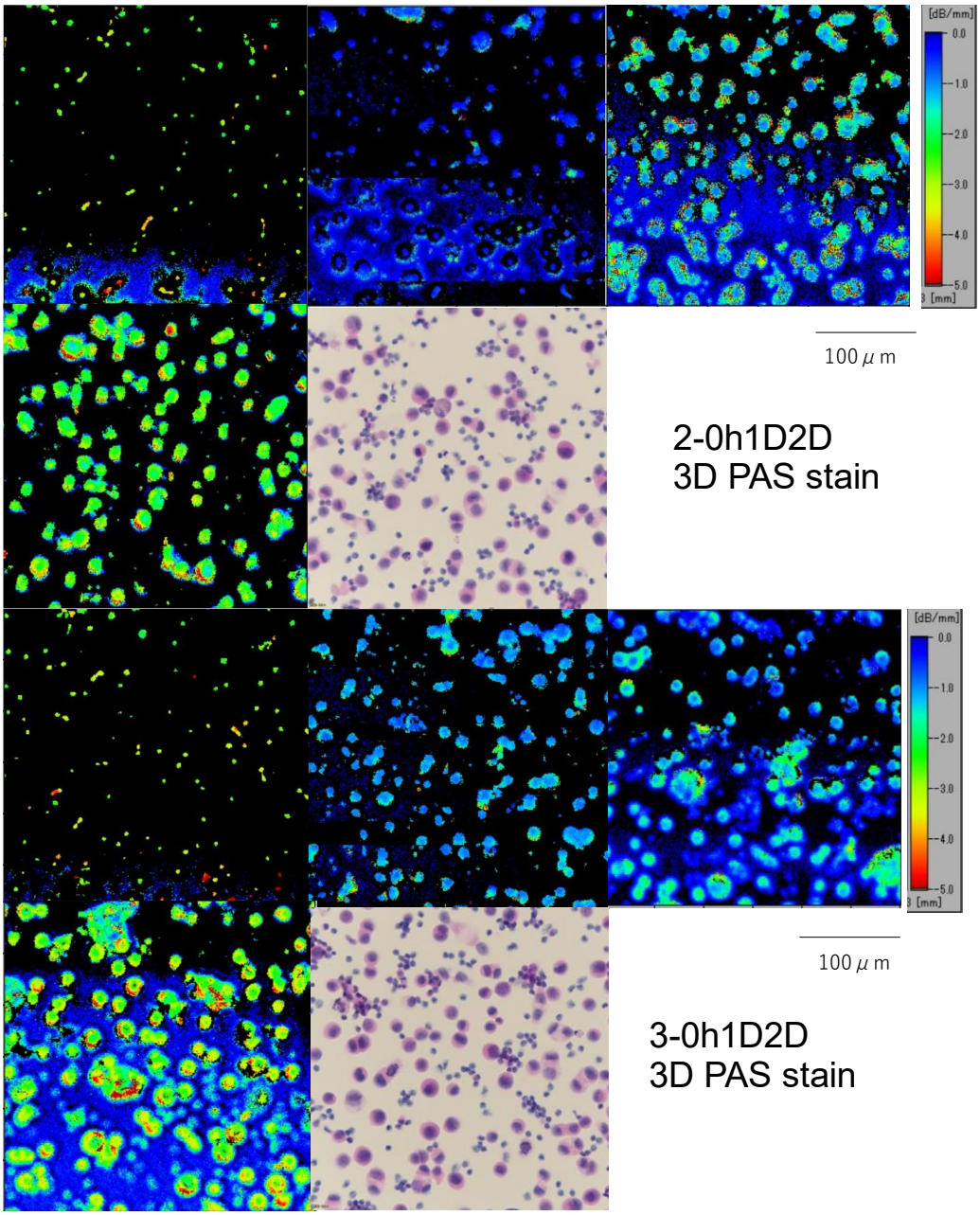


Fig. 4
AOS images of the bile duct in different conditions.
Differences in AOS images during incubation were compared over time. The corresponding PAS-stained LM images on day 3 after incubation were at the lower right. In every group, the AOS images at 0 h after incubation showed the greatest AOS values. No significant difference was observed between the four groups at any time. 1, glyceraldehyde (GA); 2, GA + 50 mM glucose; 3, GA + 100 mM glucose; 4, GA + 200 mM glucose.

Attenuation of sound (AOS) images of adenocarcinoma cells in pleural fluid during glycation were compared across different glucose concentrations (1, glyceraldehyde (GA) only; 2, GA + 50 mM glucose; 3, GA + 100 mM glucose; 4, GA + 200 mM glucose) (Fig. 5A). At 0 h, all cell groups were small in size with high AOS values. On day 1, the cells expanded and showed the lowest AOS after incubation. Thereafter, the AOS values gradually increased up to day 3. Among the four groups, those with higher glucose concentrations presented greater AOS values. The cell surface displayed the highest area in the AOS images, making ring structures. Among the PAS staining images, no conspicuous differences were found. Figure 5B shows a dot blot of the AOS values for adenocarcinoma cells (mean + standard deviation) after incubation with the four solutions. All groups gradually increased AOS values from days 1 to 3. At any time, the group with higher glucose concentrations presented greater AOS values.




[dB/mm]
0.0
1.0
2.0
3.0
4.0
5.0
[mm]
100 μ m
2-0h1D2D
3D PAS stain
[dB/mm]
0.0
1.0
2.0
3.0
4.0
5.0
[mm]
100 μ m
3-0h1D2D
3D PAS stain

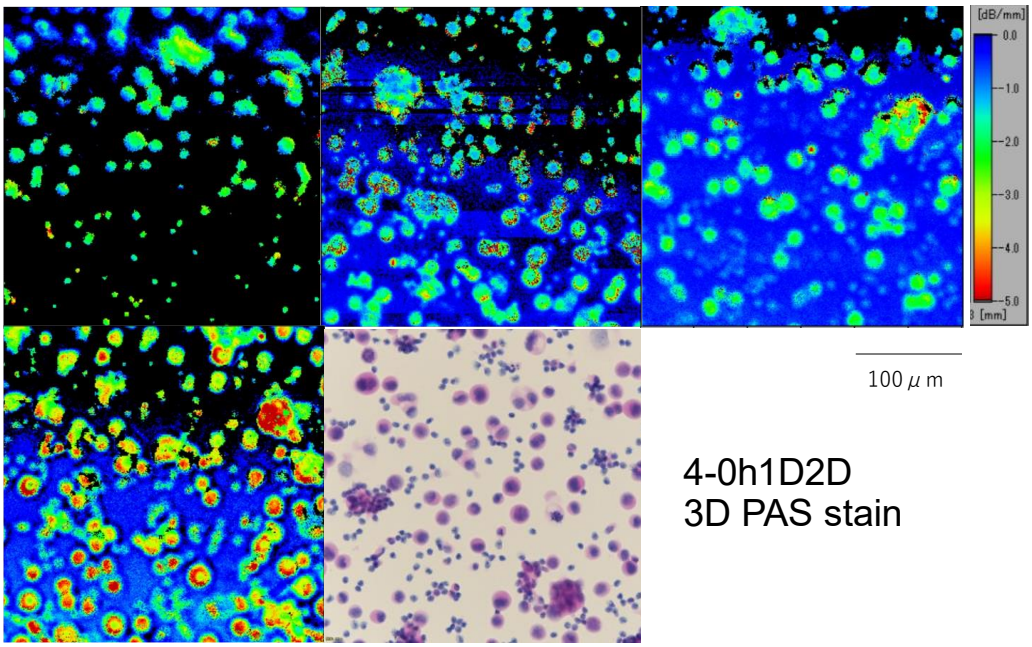


Fig. 5A

Adenocarcinoma cells in the pleural fluid were soaked in different solutions. At 0 h, the cells were small in size with high AOS values. On day 1, the cells expanded and had the lowest AOS values after incubation. Thereafter, the AOS values gradually increased up to day 3. Among the groups, those with higher glucose concentrations displayed greater AOS values. The cell surface displayed the highest area in the highest AOS images, making ring structures. The lower middle images show PAS staining results on day 3. Large PAS-positive cells are adenocarcinoma cells. Small cells are lymphocytes. 1, Glyceraldehyde (GA); 2, GA + 50 mM glucose; 3, GA + 100 mM glucose; 4, GA + 200 mM glucose

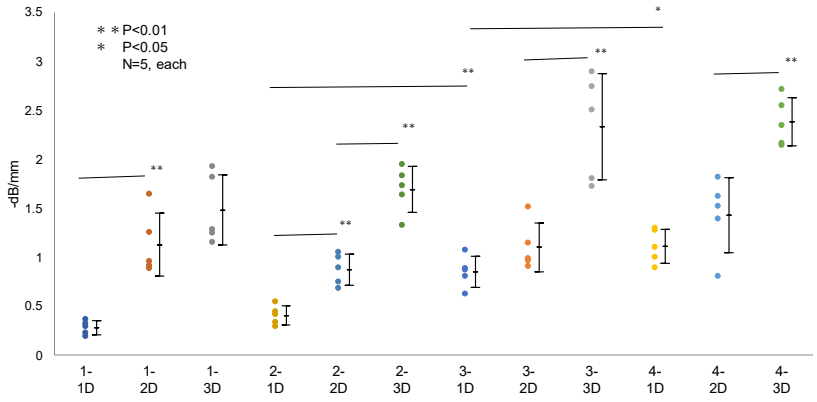


Fig. 5B

Dot blot of the AOS values for adenocarcinoma cells (mean + standard deviation) after incubation with the four solutions. All groups gradually increased AOS values from days 1 to 3. At any time, the group with higher glucose concentrations presented greater AOS.

Discussion

The current study was able to visualize GA-induced glycation with glucose supplementation using AOS alteration. Accordingly, higher glucose concentrations increased the AOS values of the sections and cells. Although GA increased AOS values after incubation (Figs. 1A–D), GA supplemented with glucose increased AOS values more than GA alone (Figs. 2–5). The aldehyde groups bind amino or guanidino groups of proteins. However, its binding is less intense and time-consuming compared to formaldehyde or glutaraldehyde, which has been used as a fixative. Glucose did not affect proteins by itself (Figs. 2A, B) but promoted progressive glycation effects in the presence of GA. Glucose concentration affected the degree of glycation (Figs. 3–5), with more glucose promoting more glycation.

Although PAS staining images showed the degree of glycation, the results were analog and unstable. AOS images are digital and can be compared to other images, which makes them suitable for statistical analysis.

The increase in AOS values varied according to organ and cell, which supports the difference in affected organs among patients with diabetes mellitus (DM). The arterial smooth muscle, collagen, and intima were apt to accept glycation, whereas the mucosa showed no remarkable alteration in the current study. This result supports prior findings showing that DM severely affects blood vessels[15,16]. In many cases, fresh tissues and cells showed decreased AOS values after incubation, which indicated that the glycation was not strong enough to compensate for tissue degeneration. Although ethanol fixation removed water in the cell, the cell expanded after incubation due to water absorption. The tissue and cell density were reduced, which promoted a decrease in AOS values.

AOS values corresponded well with glycation level. We previously reported that the reduction in AOS values correlated with amyloid fibril breakdown.[17] The present study used the increase in AOS values to indicate cross-linking. Indeed, plotting AOS values enabled the creation of histological images showing where and to what degree structures had altered over time.

Several measurements have been developed to date to determine AGEs, such as chromatographic, colorimetric, spectroscopic, mass spectrometric, and serological methods.[18] For histological detection of AGEs, antibody-based staining has been used.[19,20] However, this immunostaining method has several disadvantages, such as potential artifacts due to pretreatment steps, including heating and alkaline treatment.[19] AGEs could be generated through these steps or disappear through breakdown. Moreover, autoantibodies against AGEs may interfere with detection. The present method uses simple AOS values, which can be followed over time using the same slides. GA-induced glycation and other forms of glycation can be evaluated through the present method.

Several limitations of this study should be acknowledged. First, more tissues and cells need to be studied before these glycation results can be applied to every organ or cell. Accessibility differences among tissues can be a concern. Second, the duration of glycation in the current study spanned from 2 or 4 days. The protein life span in the human body is longer, which could lead to greater accumulation of glycation. Red blood cells, for instance, have been known to have a 120-day lifespan. Hemoglobin A1c may be influenced by glycation during this long period. Third, glyceraldehyde-induced glycation represents a single event of glycation, and glycation is a complex reaction related to many chemical components.

Conclusion

Tissue and cellular glycation induced by GA with glucose was studied using AOS values. Compared to GA alone, glucose supplementation accelerated glycation depending on the concentration of glucose. The degree of glycation differed according to tissue type. Collagen and smooth muscles were predisposed to glycation, whereas the mucosa was relatively stable. Higher glucose concentrations for an extended period of time promote greater glycation. Our results confirm that a prolonged hyperglycemic state is associated with increased glycation and formation of AGEs.

Conflicts of interest

KM received supporting fees from Honda Electronics for attending meetings. TI has no conflicts of interest to declare.

Funding

No funding was received.

Acknowledgments

The authors acknowledged Drs. Yuki Egawa and Toshiaki Moriki at Shizuoka City Hospital for the sample preparation. Drs. Kanna Yamashita, Michio Fujie, and Toshi Nagata of the Department of Health Science, Hamamatsu University School of Medicine provided the research facilities for this study. Miss Mitsue Kawashima at the Department of Regenerative & Infectious Pathology, Hamamatsu University School of Medicine, helped with the sample staining.

References

1. Monnier VM, Nagaraj RH, Portero-Otin M, et al. *IMephrology Dialysis Transplantation Structure of Advanced Maillard Reaction Products and Their Pathological Role.*; 1996. https://academic.oup.com/ndt/article/11/supp5/20/1856983
2. Chandel NS. Glycolysis. *Cold Spring Harb Perspect Biol*. 2021;13(5). doi:10.1101/CSHPERSPECT.A040535
3. Schalkwijk CG, Stehouwer CDA, van Hinsbergh VWM. Fructose-mediated non-enzymatic glycation: Sweet coupling or bad modification. *Diabetes Metab Res Rev*. 2004;20(5):369-382. doi:10.1002/dmrr.488
4. Vasdev S, Stuckless Bsc J, Adfs H. *Role of Methylglyoxal in Essential Hypertension*. Vol 19.; 2010.
5. Chellan P, Nagaraj RH. Early glycation products produce pentosidine cross-links on native proteins. Novel mechanism of pentosidine formation and propagation of glycation. *Journal of Biological Chemistry*. 2001;276(6):3895-3903. doi:10.1074/jbc.M008626200
6. Takeuchi M, Yamagishi S. TAGE (toxic AGEs) hypothesis in various chronic diseases. *Med Hypotheses*. 2004;63(3):449-452. doi:10.1016/j.mehy.2004.02.042
7. Vyas M, Zuckerman J, Andeen N, Tsang P. PAS (Periodic acid-Schiff). PathologyOutlines.com website. Published December 17, 2023. Accessed December 31, 2023. https://www.pathologyoutlines.com/topic/stainspas.html
8. Alsaad KO, Herzenberg AM. Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: An update. *J Clin Pathol*. 2007;60(1):18-26. doi:10.1136/jcp.2005.035592
9. Saijo Y. Acoustic microscopy: latest developments and applications. *Imaging Med*. 2009;1(1):47-63. doi:http://dx.doi.org/10.2217/iim.09.8
10. Azhari H. Appendix A: Typical Acoustic Properties of Tissues. *Basics of Biomedical Ultrasound for Engineers*. Published online 2010:313-314. doi:10.1002/9780470561478.app1
11. Mast TD. Empirical relationships between acoustic parameters in human soft tissues. *Acoustic Research Letters Online*. 2000;1(November 2000):37-42. doi:10.1121/1.1336896
12. Miura K, Yamamoto S. Histological imaging from speed-of-sound through tissues by scanning acoustic microscopy (SAM). *Protoc Exch*. Published online 2013. doi:10.1038/protex.2013.040
13. Tamura K, Ito K, Yoshida S, Mamou J, Miura K, Yamamoto S. Alteration of speed-of-sound by fixatives and tissue processing methods in scanning acoustic microscopy. *Front Phys*. 2023;11. doi:10.3389/fphy.2023.1060296
14. Hozumi N, Yamashita R, Lee CK, et al. Time-frequency analysis for pulse driven ultrasonic microscopy for biological tissue characterization. In: *Ultrasonics*. Vol 42. ; 2004:717-722. doi:10.1016/j.ultras.2003.11.005
15. Rask-Madsen C, King GL. Vascular complications of diabetes: Mechanisms of injury and protective factors. *Cell Metab*. 2013;17(1):20-33. doi:10.1016/j.cmet.2012.11.012
16. Creager MA, Lüscher TF, Cosentino F, Beckman JA. Diabetes and vascular disease. Pathophysiology, clinical consequences, and medical therapy: Part I. *Circulation*. 2003;108(12):1527-1532. doi:10.1161/01.CIR.0000091257.27563.32
17. Miura K, Iwashita T. Observations of amyloid breakdown by proteases over time using scanning acoustic microscopy. *Sci Rep*. 2023;13(1):20642. doi:10.1038/s41598-023-48033-4
18. Perrone A, Giovino A, Benny J, Martinelli F. Advanced Glycation End Products (AGEs): Biochemistry, Signaling, Analytical Methods, and Epigenetic Effects. *Oxid Med Cell Longev*. 2020;2020. doi:10.1155/2020/3818196
19. Nagai R, Shirakawa J ichi, Ohno R ichi, et al. Antibody-based detection of advanced glycation end-products: promises vs. limitations. *Glycoconj J*. 2016;33(4):545-552. doi:10.1007/s10719-016-9708-9
20. Crisan M, Taulescu M, Crisan D, et al. Expression of Advanced Glycation End-Products on Sun-Exposed and Non-Exposed Cutaneous Sites during the Ageing Process in Humans. *PLoS One*. 2013;8(10). doi:10.1371/journal.pone.0075003