Authentication of In Situ Measurements for Thoracic Aortic Aneurysms in Mice

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Running Title: OCT injection and in situ aortic imaging in mice

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Abstract

Aortic diameter is a standard parameter for defining disease severity of thoracic aortic aneurysms. In mouse studies, aortic diameters can be measured directly in situ, but this approach has the potential confounder of underestimation due to the absence of physiological arterial pressure. In the present study, we developed an in situ approach for authentic aortic measurements. Thoracic aortic aneurysms were induced by β-aminopropionitrile (BAPN, 0.5% wt/vol) administration in 4-week-old male C57BL/6J mice. Ultrasonography was performed to examine aortic dimensions, and mice with thoracic aortic dilatations were terminated subsequently. After saline perfusion through the left ventricle, periaortic tissues were removed to expose thoracic aortas. Optimal cutting temperature (OCT) compound was injected via the left ventricle to maintain aortic patency. In situ aortic images were captured pre- and post-OCT injection. In mice with severe thoracic aortic aneurysms, smaller aortic diameters were observed prior to OCT injection compared to ultrasound measurements, while aortic diameters in situ after OCT were comparable to diameters measured using ultrasound. Immunostaining for CD31 revealed that endothelial cells were preserved in the intima after OCT injection, indicating that OCT injection does not cause endothelial damage. In conclusion, in situ imaging with OCT injection provides authentic aortic measurements without overt aortic damage in mice with thoracic aortic aneurysms.

In thoracic aortic aneurysms, aortic dimensions are commonly measured as a criterion of disease severity.[1] With the advent of high frequency ultrasound instruments, aortic diameters can be determined in vivo by ultrasonography in mice.[2, 3] However, acquisition of measurements using this expensive instrument is dependent on operator's skill and standardization of procedure. To verify ultrasound measurements and as an alternative approach in the absence of this modality, aortic dimensions can be measured directly in situ.[4] A potential caveat of aortic measurements using this mode is the absence of physiological arterial pressure that underestimate in situ dimensions, particularly in mice with a flaccid aortic wall. To overcome this shortcoming, we developed an in situ imaging approach for acquiring authentic aortic dimensions in mice.

β-aminopropionitrile (BAPN, 0.5% wt/vol in drinking water) was administered to induce thoracic aortic aneurysms in C57BL/6J mice (male, 4-week-old, n=30).[5] Ultrasound imaging (Vevo 2100, MS550) was performed after 4, 8, and 12 weeks of BAPN administration, and aortic diameters were measured at the most dilated area at end diastole. BAPN administration resulted in a wide range of luminal dilatations of the thoracic aorta from mild to severe as measured by ultrasound (**Figure A, B, Supplemental Figure**). Mice with aortic aneurysms were subsequently terminated (n=7). The right atrial appendage was excised and saline (8 ml) was perfused through the left ventricle. Perivascular tissues of the thoracic aorta were removed gently and a black plastic sheet was inserted behind the aorta to enhance contrast of the aortic wall. Optimal cutting temperature (OCT) compound (150 µl) was then introduced into the left ventricle using an insulin syringe to maintain aortic patency. Two dimensional images and cine loops were recorded from pre-injection to 50 seconds post OCT injection using a dissection microscope with a high-resolution camera (#SMZ800, #DS-Ri1, Nikon). Aortic diameters were compared between ultrasound and in situ images. Mice with severe dilatations exhibited smaller aortic diameters in images acquired prior to introduction of OCT compared to diameters measured using ultrasound (−0.8 to −0.4 mm). OCT injection inflated the aortic dimensions (**Figure A, B, Supplemental Figure**). A Bland-Altman plot demonstrated that diameters after OCT injection were comparable to diameters measured by ultrasound (**Figure C**), and post-OCT aortic diameters were correlated positively with luminal diameters measured by ultrasonography (**Figure D**). Therefore, in situ imaging using OCT injection provided more accurate aortic measurements in mice with thoracic aortic aneurysms.

OCT was injected using a small gauge needle (28G) that provided a slow flow of this viscous material. Thus, OCT injection was not expected to cause aortic damage. To assuage this concern, the integrity of the aortic wall was determined during OCT injection. Aortic diameters were measured during OCT injection in mice with severe aortic dilatation (n=3). Aortic diameters were the largest immediately following OCT injection, with a subsequent modest reduction that was plateaued after at 40 seconds (**Figure E, Supplemental Video**). Importantly, maximal external diameters measured with in situ images were comparable to luminal diameters at mid systole in ultrasound images (ultrasound: 3.0±0.4; in situ: 3.0±0.4 mm, p=0.70). In addition, endothelial cells were examined by immunostaining for CD31 to determine whether OCT injection led to

loss of endothelial cells. Aortic tissues were harvested from C57BL/6J male mice after OCT injection (n=5). CD31 positive cells were detected throughout the intima of both ascending and descending thoracic aortas (**Figure F**). These results demonstrated that OCT injection did not cause overt aortic tissue damage.

Aortic patency is often not maintained during ex vivo imaging of aneurysmal aortas, which may lead to underestimation of aortic dimensions. Therefore, formalin or latex perfusion are frequently utilized to recapitulate aortic morphology ex vivo.[6-8] However, these procedures need a perfusion system and are time-consuming. OCT is a nonhazardous reagent and can be readily applied. In the present study, OCT injection provided comparable measurements of aortic diameters to ultrasonography. In addition, OCT did not cause discernable tissue damage. Therefore, OCT injection is a simple and effective procedure for accurate in situ aortic measurements. Notable, in situ images measure aortic diameters in a lateral (right-left) axis. Imaging planes should be optimized individually, especially mice with significant aortic dilatation on the anterior or posterior wall.

In conclusion, in situ imaging with OCT injection enables the acquisition of authentic measurements of aortic dimension and permits more accurate determination of thoracic aortic aneurysm severity in mice.

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Disclosure
None

Materials and Methods

All raw data and analytical methods are available from the corresponding author upon appropriate request.

Mice

C57BL/6J male mice at 3 weeks of age were purchased from The Jackson Laboratory (Stock# 000664, Bar Harbor, ME). Mouse housing conditions are described in **Table 1**. Mice were housed 5 per cage and allowed access to diet (Teklad Irradiated Global 18% Protein Rodent Diet, Cat# 2918, Envigo, Madison, WI) and water *ad libitum*. Bedding was changed weekly during the study, and cotton pads were provided as enrichment. The room was maintained at a 14:10 hour light:dark cycle, constant temperature of 18-23 °C, and 40-60% humidity. All protocols were approved by the University of Kentucky IACUC.

Table 1. Mouse Housing Conditions

Parameter	Conditions	Note
Housing density	5 per cage	
Water	0.5% BAPN mixed Water	ad libitum
Diet	Teklad Irradiated Global 18% Protein Rodent Diet, Cat# 2918; Envigo	ad libitum
Bedding	P.J. Murphy Coarse SaniChip Cotton pads for enrichment	
Light Cycle (light:dark)	14:10 hours	
Set Temperature range	18-23 °C	
Set Humidity range	40-60%	

Induction of thoracic aortic aneurysms

β-aminopropionitrile (0.5% wt/vol, Cat# A3134, Millipore-Sigma, St. Louis, MO) was administered through drinking water at 4 weeks of age.[5] BAPN drinking water was replaced twice a week.

In vivo and in situ evaluation of thoracic aortic aneurysms

Thoracic aortas were scanned at 4, 8, and 12 weeks of BAPN administration using a Vevo 2100 ultrasound system with a MS 550 transducer (FUJIFILM VisualSonics Inc., Canada). During ultrasound scanning, mice were placed on a heated platform (37°C) to avoid hypothermia and anesthetized by isoflurane (1-2% vol/vol) to adjust the heart rate between 400 to 550 beats/minutes. Longitudinal images of ascending and descending aortas were captured in the parasternal or paraspinal approach respectively, as described previously.[2, 3] Aortic diameters were measured at end diastole in 3 different cardiac cycles. Ultrasound measurements were performed using Vevo LAB 3.1.1 software (FUJIFILM VisualSonics Inc.).

Mice in which ultrasound detected aortic dilatation were euthanized at the same day or one day after ultrasonography by ketamine:xylazine cocktail (90, 10 mg/kg, respectively). The right atrial appendage was excised and saline (8 ml) was perfused via the left ventricle. Periaortic tissues were removed gently and a black plastic sheet

was then inserted under the aorta. Thoracic aortas were inflated by injection of optimum cutting temperature compound (150 µl, OCT, Cat# 23-730-571, Thermo Fisher Scientific, Waltham, MA) from the left ventricle using an insulin syringe with 28-gauge needle. In situ aortic images were captured before, and 50 seconds after, OCT injection with a Nikon SMZ800 stereoscope (Nikon, Tokyo, Japan). Cine loops were also recorded during OCT injection. All aortic in situ images were captured with a small ruler placed next to the aorta. Aortic images were analyzed using NIS-Elements AR software (Ver 4.51, Nikon). The measurement software was calibrated using the ruler on individual images. To measure aortic diameters, a measurement line was drawn perpendicularly to the aortic axis at the most dilated area. Aortic measurements were verified by an independent investigator who was blinded to the initial analysis.

Immunostaining

Ascending and descending aortas were harvested and fixed with paraformaldehyde (4% wt/vol) overnight followed by 24 hours incubation with sucrose (30% wt/vol). Aortic samples were then embedded in OCT and cut into 10 µm sections. Slides were baked at 60 °C for 20 minutes and immersed in H_2O_2 (1% vol/vol) in methanol to quench endogenous peroxidase. Sections were incubated with normal goat serum (2.5% vol/vol) for 20 minutes. Rat anti mouse CD31 (1 µg/ml, Cat# ab7388, abcam, Cambridge, MA) was used as the primary antibody. Detection of the primary antibody was visualized using Rat ImmPRESS (Cat# MP-7444, Vector Laboratories, Burlingame, CA) and ImmPACT AEC kits (Cat# SK-4205, Vector Laboratories). Tissue sections were counterstained subsequently with hematoxylin. Histological images were captured using an Eclipse E600 microscope with a DSRi1 digital camera (Nikon).

Statistical analyses

SigmaPlot 14.0 (SYSTAT Software Inc., Palo Alto, CA) was used for statistical analyses. All data were expressed as mean ± standard error of the mean. Normality and homogeneous variance were confirmed in all data by Shapiro-Wilk and Brown-Forsythe tests, respectively. Correlations between in vivo and in situ aortic diameters was evaluated by Pearson correlation coefficient. The transition of aortic diameters during OCT injection was examined by one-way repeated measures ANOVA after square root transformation. $P<0.05$ was considered statistically significant.

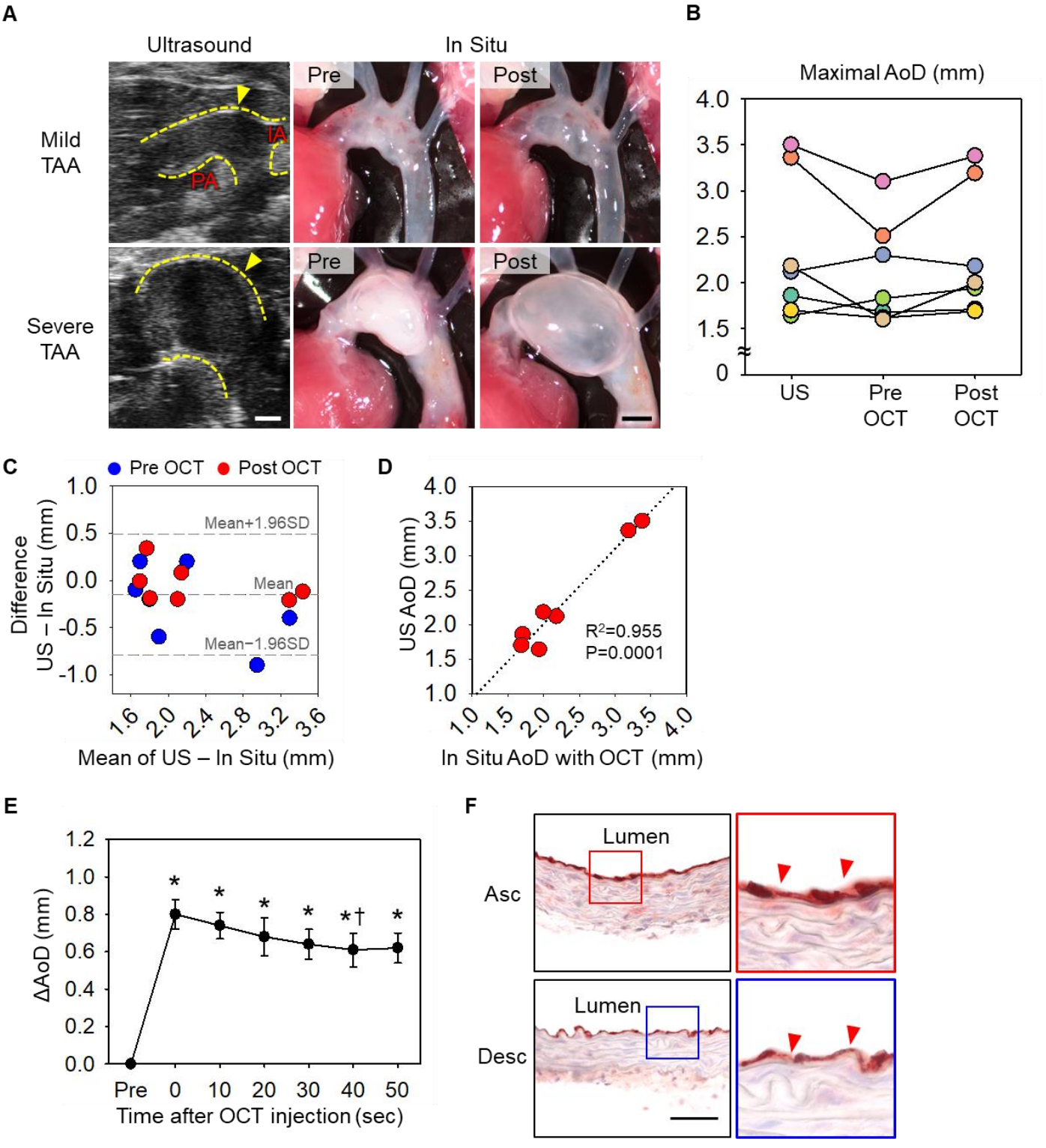

Figure. Ultrasound and in situ imaging of the thoracic aorta in mice.
(A) Representative ultrasound and in situ images of thoracic aortas in BAPN-administered mice. Post OCT images were captured at 50 seconds after OCT injection. Yellow dotted lines indicate the aortic wall; IA, innominate artery; PA, pulmonary artery. Scale bar, 1 mm. n=7. **(B)** Aortic diameters measured at the most dilated area of ultrasound and in situ images with/without OCT injection. n=7. **(C)** Bland-Altman plot

reveals the low variation of aortic measurements with OCT injection. **(D)** Significant positive correlation of aortic measurements between ultrasound and in situ with OCT. **(E)** Sequential transition of aortic diameters after OCT injection in aneurysmal tissues. n=3. * P<0.05 vs Pre, † P<0.05 vs 0 sec by one-way repeated measures ANOVA with Bonferroni t-test. **(F)** Immunostaining for CD31 of aneurysmal aortas with OCT injection. n=5. Red triangles indicate endothelial cells; L, lumen; Asc, ascending aorta; Desc, descending; scale bar, 50 μm.

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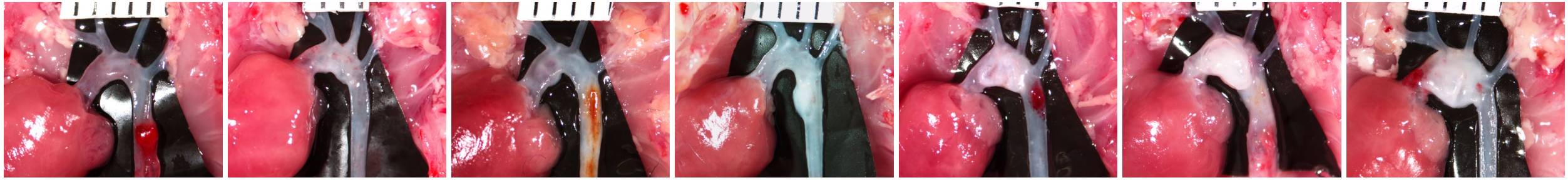
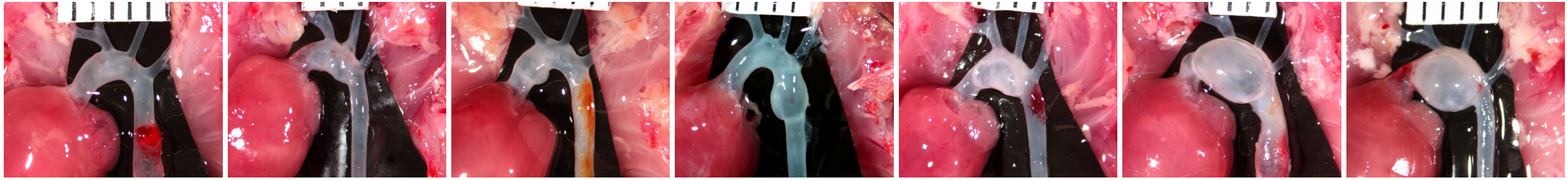
(A) Pre OCT



(B) Post OCT



Mild → Aneurysms → Severe

Supplemental Figure. In situ images of **(A)** pre- and **(B)** post-OCT injection for thoracic aortic aneurysms in BAPN-administered mice. Images are placed in order of the severity of thoracic aortic aneurysms from mild to severe.