Tracking cardiovascular comorbidity in models of chronic inflammatory disease

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Abstract

Immune-mediated inflammatory diseases (IMIDs) are commonly associated with complex co-existing conditions, and cardiovascular comorbidities are a common cause of mortality in systemic inflammation. Experimental models of disease provide an opportunity to dissect inflammatory mechanisms that promote damage to vascular tissues affected by comorbidity. Here, we describe methods to recover the thoracic aorta from mice during experimental inflammatory arthritis and assess vascular constriction responses by isometric tension myography. To support assessment of functional changes in the vasculature during inflammatory arthritis, we also outline a method to characterize vascular inflammation by immunohistochemistry.

Key words; vascular contractile dysfunction, isometric tension myography, histopathology, inflammatory arthritis, rheumatoid arthritis, cardiovascular comorbidity.

Running head; Tracking cardiovascular comorbidity in chronic inflammation

1 Introduction

Immune-mediated inflammatory diseases (IMIDs) often involve complex clusters of co-existing conditions that impact the wellbeing, quality of life, disability and mortality of patients. Comorbidity and multimorbidity refer to the existence of two or more chronic diseases in the same individual. Recent evidence suggests that the incidence of autoimmunity is increasing and, consequently, multimorbidity is forecast as an emerging global health challenge ***(1-3)***. Traditional therapeutic strategies focus on treating primary 'index' diseases rather than complex clusters of conditions that co-exist. Reflecting this, research into conditions that occur concurrently is often performed separately. There is, therefore, a pressing need to understand disease mechanisms that link chronic systemic inflammation and tissue-specific autoimmunity with co-existing damage that occurs in tissues affected by comorbidity. Experimental models of disease involving animals represent an opportunity to investigate the mechanistic basis of co- and multi-morbidity, thus complementing observational studies in human disease.

Cardiovascular comorbidity is a common cause of morbidity and mortality in patients with IMIDs such as inflammatory arthritis (e.g., rheumatoid arthritis) and diabetes (type 1 and type 2 diabetes mellitus) ***(4-6)***. While systemic inflammation is recognized as a key driver of cardiovascular disease risk, the mechanisms involved remain unclear. Experimental models of rheumatoid arthritis including collagen-induced arthritis (CIA) and antigen-induced arthritis (AIA) provide a platform to investigate shared immune mechanisms that drive co-existing joint pathology and cardiovascular dysfunction ***(7-9)***). The well-characterized time course of disease in models of rheumatoid arthritis allows investigation of cardiovascular inflammation and dysfunction at time points that reflect pre-symptomatic, early and established phases of the disease ***(10-12)***. Immunodetection methods such as immunohistochemistry, immunofluorescence microscopy and flow cytometry provide a way to quantify and characterize inflammatory changes in tissues of the cardiovascular system. From a functional perspective, the vasculature serves as a conduit for nutrient exchange, carries oxygenated blood to tissues, and the contractile state of arteries and arterioles regulate blood pressure. Here, vascular smooth muscle cells in the arterial wall mediate vasoconstriction and relaxation, and isometric tension myography provides a sensitive assay to measure vascular function of large arteries in animal models ***(10, 11, 13, 14)***. This method involves passing wires through the lumen of blood vessels, stretching the vessel to model physiological tension and measuring changes in vascular tension in response to physiological or engineered vasoconstrictors ***(14)***. Comparing the contractility of vessels during health and chronic disease provides insight into the effect of systemic inflammation on vascular function.

Here, we outline protocols for the recovery of the thoracic aorta to measure vascular constriction responses in mice with CIA. To complement the analysis of vascular function, a protocol to characterize local immune changes by immunohistochemical analysis of the aorta and surrounding perivascular adipose tissue (PVAT) is provided. These methodologies can be extended to investigate vascular function in other disease models where cardiovascular comorbidities feature. Together, these methods serve to identify mechanisms that underpin vascular dysfunction in inflammatory arthritis and provide an *in vivo* platform to test novel drugs for cardiovascular comorbidities in IMIDs.

2 Materials

Unless otherwise stated, all solutions and buffers should be prepared in distilled water.

2.1 Animal Ethics

Animal experiments should be performed with ethical consent from local and national authorities. In the United Kingdom, experiments involving animals comply with the Animals (Scientific Procedures) Act 1986 and are performed under the authority of Personal Licenses, Project Licenses and Establishments Licenses. Experiments should be designed, performed and reported with good practice, incorporate refinements that improve animal welfare, and use the minimum number of mice needed to achieve scientifically valid data (see **Note 1**).

2.2 Recovery of the thoracic aorta and isometric tension myography

1. Mice from an approved supplier for scientific research and induced with an experimental disease model (see **Note 2**).
2. Dissection kit containing scissors, high-precision tweezers and micro-scissors.
3. Dissection board (e.g., cork or polystyrene matt).
4. Dissection lamp.
5. Disposable 1 mL syringes and needles (25G x 16mm).
6. Krebs buffer: 109 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM $C_6H_{12}O_6$, 1.7 mM $CaCl_2$. Freshly prepared, to maintain tissue viability for myography.
7. High potassium Krebs buffer: 39.3 mM NaCl, 60 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM $C_6H_{12}O_6$, 1.7 mM $CaCl_2$. Freshly prepared for myography.
8. Dissection microscope to carefully remove PVAT from the aorta.

9. Wire Myograph System. For example, the Multi Myograph model 620M by Danish Myo Technology (DMT) with heating block, gas supply (95% oxygen, 5% CO_2), and compatible acquisition software (e.g., LabChart by ADInstruments, DMT Myodaq).
10. 5-hydroxytryptamine (5-HT): a range of concentrations from 1 x 10^{-9} to 3 x 10^{-5} mM (see **Table 1** and **Note 3**).
11. 8% (v/v) acetic acid to clean the myograph organ baths.

2.3 Immunohistochemistry

1. 10% (v/v) formalin solution, neutral buffered.
2. Tissue processing and heated paraffin embedding stations for histological processing of tissue.
3. High adhesion microscope slides (e.g., SuperFrost Plus).
4. Microtome with a blade suitable for hard tissue (e.g., FEATHER R35 blade)
5. Paraffin wax.
6. Xylene.
7. 70% (v/v), 90% (v/v) and 100% (v/v) alcohol (e.g., industrial methylated spirits).
8. Sodium citrate buffer for antigen retrieval: 10 mM sodium citrate, 0.05% (v/v) Tween 20, pH 6.0
9. Hydrophobic barrier pen (e.g., ImmEDGE™ pen from Vector Laboratories)
10. 3% hydrogen peroxide solution, freshly prepared from 30% (w/w) hydrogen peroxide solution in H_20 (see **Note 4**).
11. Humidified staining chamber for immunohistochemistry.
12. Normal serum for blocking (see **Note 5**). Herein, normal swine serum is used.
13. Tris Buffered Saline containing Tween 20 (TBS/T): 50 mM Tris base, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20, pH 8.4.
14. Primary antibody specific to antigen of interest. Herein, polyclonal rabbit anti-human/mouse CD3 is used (A0452 Dako).
15. Biotinylated secondary antibody against primary antibody. Herein, a biotinylated polyclonal swine anti-rabbit IgG is used.
16. Avidin-biotin with horseradish peroxidase (HRP)-based detection system (e.g., VECTASTAIN ELITE ABC Kit from Vector Laboratories).
17. Harris hematoxylin.
18. HRP substrate kit (see **Note 6**): 3,3'-diaminobenzidine (DAB).
19. DPX mountant.

3 Methods

3.1 Recovering the thoracic aorta from *in vivo* models of chronic inflammatory disease

1. Kill mice using an approved humane method at an appropriate time point during a model of chronic inflammation. In the example given here, the aorta is recovered during AIA, which is initiated following published protocols ***(7)*** (see **Note 7**).
2. Laying the mouse on its back, stretch the four limbs perpendicular to the body axis and pin the paws to a dissection board using syringe needles to stabilize and expose the ventral side.
3. Make a midline incision using dissection scissors from the base of the abdomen to the top of the thorax. To expose the abdominal and thoracic cavities, pull back the skin from the underlying tissue and pin to the dissection board using syringe needles. Make a midline incision along the abdominal cavity to the sternum, removing the peritoneal membrane to expose the internal organs.
4. Cut into the sternum and the ribs along the midline, removing the upper ventral side of the rib cage. This will expose the heart.
5. Using a 1 mL syringe and 25G needle inserted into the left ventricle of the heart, gently and slowly perfuse 1 mL of Krebs solution to remove blood from the aorta.
6. Remove the intestines, stomach, spleen, liver, lungs and diaphragm. This will provide a clear view of the heart and aorta surrounded by PVAT (**Fig. 1A**).
7. Excise the heart at the aortic arch. Remove excess blood gently using a tissue.
8. Using high-precision tweezers, micro-scissors and a dissection lamp if needed, tightly hold the aorta with the tweezers at the base of the thoracic cavity. Taking care not to tear or stretch the aorta, gently cut the aorta and surrounding PVAT away from the spine by moving up the chest cavity while holding the bottom of the aorta with the tweezers (**Fig. 1B-D**). Cut at the point where the aorta meets the aortic arch.
9. Keep the aorta in Krebs buffer until ready to start the myography.

3.2 Preparing the aorta for isometric tension myography

1. Place the aorta in a petri dish containing Krebs buffer (see **Note 8**).
2. Using high-precision tweezers and micro-scissors, gently remove the PVAT from the aorta under a dissection microscope at the desired magnification (typically 5X), taking care not to cut or damage the aorta wall **(Fig. 2A)**.
3. Fill the myograph organ bath with Krebs buffer so that the mounting pins are submerged. Adjust the mounting pins into a closed position using the tension control dial (**Fig. 2B** and **Fig. 2E-F**).

4. With the PVAT removed, cut a 2-5 mm section of the aorta and gently slide around the two mounting pins of the myograph (**Fig. 2B**) (see **Note 9**).
5. Manually open wire pins containing the aortic ring using the tension control dial so that enough tension is applied to hold the aorta in place, being careful not to stretch the aorta (**Fig. 2C**).
6. Place the organ bath onto the heated base of the myograph and connect the gas supply (95% oxygen, 5% CO_2) (**Fig. 2D-H**).
7. Adjust the tension to zero and start data acquisition using appropriate software to record the baseline tension (see **Note 10**).

3.3 Measuring vascular constriction responses by isometric tension myography

1. Allow the mounted aorta to equilibrate for about 20 min then gradually open the wire pins, increasing the baseline tension to 5 mN (see **Note 11**). To do this, manually increase the tension by 0.5 mN increments every minute on the minute. Once set to 5 mN, allow to equilibrate for 20 min.
2. Depolarize the vascular smooth muscle cells by adding high potassium Krebs buffer and incubate for 20 min.
3. Remove the high potassium Krebs buffer and thoroughly wash in 3 changes of Krebs buffer. Allow to re-equilibrate and return to baseline tension in Krebs buffer for 20 min (see **Note 12**).
4. Prepare concentrated solutions of 5-HT to add to the myography bath for measuring thoracic aortic constriction responses (see **Table 1** for recommended final concentrations of 5-HT) (see **Note 13**).
5. After tissues have returned to baseline tension for 20 min, add the lowest concentration of 5-HT and mark its addition on the Myodaq software. Allow the vessel to equilibrate for 5 minutes before adding the next dose of 5-HT.
6. Continue adding the increasing concentrations of 5HT in half-log increments to the myography bath. Only move to the next 5-HT concentration when tension has plateaued and note the concentration of 5-HT used at each addition.
7. Export the data or note the maximum tension recorded after each addition of 5-HT. Generate a graph showing the contraction response curve to 5-HT concentrations by plotting Developed Tension (mN) on the y-axis and Log 5-HT concentration on the x-axis (**Fig. 2I**).

3.4 Histological processing of the aorta

1. Recover the aorta from mice as described in section 3.1, placing the aorta with intact PVAT into 10% (v/v) neutral buffered formalin solution for 48 hours (see **Note 14**).

2. Process the tissue into paraffin wax using a histopathology service. A suitable tissue processing cycle involving dehydration in alcohol, clearing in xylene and infiltration by paraffin wax is provided in **Table 2**.
3. Aortas should be embedded in paraffin at the orientation of interest (typically to generate cross-sections) using an embedding station and the blocks allowed to cool for 2-3 hours on a cold plate before storing at room temperature.
4. Prepare cross-sections of 5-7 µm using a microtome, placing sections onto high-adhesion microscope slides. Place slides into a 55˚C oven for 5-6 hours or overnight to dry slides and ensure strong tissue adherence.

2.3.6 Immunohistochemistry

1. Dewax formalin fixed paraffin embedded (FFPE) aorta sections in three changes of xylene for 5 min each, followed by rehydration through decreasing concentrations of 100% (v/v), 90% (v/v) and 70% (v/v) alcohol, each for 5 min. Transfer to distilled water.
2. Perform heat-induced antigen retrieval by placing slides in sodium citrate buffer prewarmed to 95°C in a water bath (see **Note 15**). Incubate at 95˚C for 40 min then remove from water bath and allow to cool at room temperature for 30 min.
3. Using a hydrophobic barrier pen, draw a circle around each tissue section to form a barrier that keeps staining reagents on the tissue and to prevent the mixing of staining reagents when multiple tissue sections are present on a single slide.
4. Cover each tissue section with 3% hydrogen peroxide solution (see **Note 4**). For aorta cross-sections, 100-200 µl is sufficient. Incubate at room temperature for 10 min in a humidified staining chamber for immunohistochemistry (see **Note 16**).
5. Wash slides in distilled water for 5 minutes.
6. Add enough 10% (v/v) swine serum diluted in TBS/T to cover each tissue section. Incubate for 1 hour at room temperature in a humidified staining chamber.
7. Wash slides in TBS/T for 5 minutes (see **Note 17**).
8. Add primary antibody, diluted in TBS/T, to the tissue section and incubate at 4˚C overnight in a humidified staining chamber (see **Note 18**). Antibody dilution should be according to the manufacturer's recommendation and may need to be optimized for different tissues. For IHC staining presented in **Fig. 3**, rabbit anti-human/mouse CD3 was used at 3 µg/ml.
9. Wash slides in three changes of TBS/T, each for 5 minutes.
10. Add biotinylated secondary antibody, diluted in TBS/T, to the tissue section. Incubate for 1 hour at room temperature in a humidified staining chamber. Antibody dilution should be according

to the manufacturer's recommendation. For IHC staining presented in **Fig. 3**, biotinylated swine anti-rabbit IgG was used at 3 µg/ml.

11. Wash slides in three changes of TBS/T, each for 5 minutes.
12. Incubate tissue with an avidin-biotin with HRP detection system following manufacturer's recommendations (e.g., VECTASTAIN ELITE ABC Kit from Vector Laboratories).
13. Wash slides in three changes of TBS/T, each for 5 minutes.
14. Incubate tissue with a HRP substrate system. For IHC staining presented in **Fig. 3**, DAB was used according to the manufacturer's instructions. Monitor the development of brown precipitate at sites of antigen-antibody binding under the microscope, typically for 2-3 minutes. When a clear brown stain is observed, stop the reaction by immersing the slides in distilled water.
15. Counter stain the tissue in Harris hematoxylin for 10-30 seconds. Wash excess stain under running tap water.
12. Dehydrate tissue sections by immersion in 70%, 90% and 100% alcohol for 5 min each. Clear in three changes of xylene for 5 min each. Place coverslips on the slides using DPX mountant.

4 Notes

1. The National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs) provides an excellent resource library of guidance and tools to support research involving animals (see https://www.nc3rs.org.uk/). In particular, the Experimental Design Assistant (EDA) tool and website (https://eda.nc3rs.org.uk/experimental-design) support sample size calculations, recommend appropriate statistical tests and incorporate approaches to reduce bias ***(15)***. For more complex power calculations involving more than 2 groups or requiring non-parametric analyses, G*Power is available ***(16)***. The ARRIVE guidelines (https://arriveguidelines.org/) provide a checklist of recommendations to improve the standard of reporting of research involving animals in publications ***(17)***.
2. The choice of genetic background and the sex of mice will be determined by susceptibility to the disease model being investigated. Tracking vasculopathy as outlined herein has been performed in the CIA model using male DBA/1 (MHC class II haplotype I-A^q) mice and the AIA model in male C57BL/6 (MHC class II haplotype I-A^b) mice ***(10, 11)***. The kinetics of arthritis onset, disease severity and co-existing vasculopathy will vary in alternative CIA and AIA susceptible strains, female mice and other disease models ***(9, 18-20)***.
3. 5-hydroxytryptamine (5-HT), also called serotonin, is a neurotransmitter and vasoconstrictor. Vascular contraction is measured against a range of 5-HT doses to generate a concentration response curve.

4. Hydrogen peroxide is used to block endogenous peroxidase activity present in some tissues. This step prevents non-specific background staining when using the combination of HRP-conjugated secondary antibodies and HRP substrates.
5. Serum is a commonly used blocking agent to prevent non-specific antibody binding. A good guide is to use serum of the same species as the secondary antibody. Alternatively, many serum-free protein blocking reagents are available commercially.
6. The DAB chromagen forms a brown precipitate in the presence of HRP. Alternative HRP substrates are available that form different colour reaction products and allow for the detection of multiple antigens in the same tissue section.
7. Mice may be killed and death confirmed using an approved method set out in Schedule 1 of the Animals (Scientific Procedures) Act 1986, for example, exposure to CO_2 gas in a rising concentration or overdose of an appropriate anesthetic agent. We have assessed vasculopathy in mice undergoing CIA and AIA. The investigator should consider the appropriate time point and model for their study. For example, vascular changes are observed at time points that model the very early stages of arthritis onset ***(10, 11)*** and in other chronic disease models including diabetes ***(13)***. The experimental design may benefit from a parallel analysis of aortas recovered from age- and sex-matched healthy naïve mice as a control group.
8. Keep the aorta in Krebs buffer throughout preparation to maintain tissue integrity for myography.
9. Once the methodology is optimized, always cut aortic rings from just below the aortic arch as this is the most muscular area and will provide a maximal tension response. Be consistent with the length of aorta used across experimental groups to ensure robust data are generated. To avoid damaging the aorta when positioning around the mounting pins, the organ bath can be removed from the myograph and placed under a dissection microscope to help visualize the careful maneuvering of the vessel.
10. When manually setting the myograph tension to zero, this may need to be repeated a few times until the tension remains stable.
11. A tension of 5 mN is recommended for the aorta as some stretch allows for optimal contact of the muscle filaments with the wire pins while retaining enough room for the vessel to contract optimally. Other vessels and those from different species may require different baseline tensions ***(14)***.
12. If after 20 min the baseline tension has fallen below 5 mN, gradually increase the tension in 0.5 mN increments as performed in section 3.3, step 1.

13. Solutions should be at least 100X the final concentration of 5-HT required in the myography bath to avoid fluctuations in temperature with the addition of each increasing concentration of 5-HT solution.
14. Over-fixation of FFPE tissues can make tissue sectioning difficult. After 48 hours, for longer term storage the tissue should be transferred to 70% (v/v) alcohol (e.g., ethanol or industrial methylated spirits) until ready for tissue processing.
15. Antigen retrieval is performed to unmask antigenic sites within tissue sections by breaking protein cross-links that form during formalin fixation. This enhances the accessibility of antibodies to previously masked epitopes improving staining intensity. Alternative antigen retrieval methods include proteolytic-induced antigen retrieval (e.g., Trypsin or Proteinase K treatment) and should be optimized for the antigen and primary antibody being investigated.
16. Use of a humidified chamber is important to avoid the tissue sections drying out, which results in non-specific staining.
17. For antigen detection where background staining is an issue, additional blocking steps can be introduced here. For example, commercial avidin-biotin blocking systems are available to block endogenous biotin, biotin receptors and avidin binding sites within tissues.
18. A negative control tissue section should also be prepared. Total IgG or a matched isotype of IgG from the same species as the primary antibody should be used.

Acknowledgements

This work was supported by funding from Versus Arthritis (reference 22706). SE is supported by a Fight for Sight and Versus Arthritis jointly funded PhD scholarship (reference 5149 / 5150) and ASM by an industry sponsored PhD studentship from Galecto Biotech AB.

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Figure legends

Figure 1 Recovery of thoracic aorta from mice

(**a**) Image of the aorta surrounded by PVAT following removal of the ventral rib cage, intestines, stomach, spleen, liver, lungs and diaphragm . The aorta can be seen lying on top of the spine. (**b**) Image showing forceps holding the aorta at the base of the thoracic cavity, where the diaphragm is located. (**c-d**) The aorta is removed gently by cutting the aorta and surrounding PVAT from the spine using micro-scissors, while maintain a hold on the base of the aorta. Continue moving up spine until the aortic arch is reached. The aorta can then be cut cleanly using the dissection scissors and transferred to Krebs buffer.

Figure 2 Overview of isometric tension myography

(**a**) After removal of the aorta from the mouse, PVAT is dissected from the aorta using a fine tweezers and micro-scissors being careful not to cut or stretch the walls of the aorta. (**b**) With the PVAT removed, a 2-5 mm section of aorta is cut and placed onto the closed mounting pins of the myograph. (**c**) The mounting pins are manually opened to hold the aorta in place without stretching the vessel. (**d**) Photograph of a Multi Myograph model 620M by Danish Myo Technology (DMT). (**e-h**) Schematic (**e**) and photographs (**f-h**) of a myograph chamber showing the heated base, gas supply, organ baths, mounting pins and tension control. (**i**) Graph showing a defective vascular constriction response to 5-HT measured by isometric tension myography in mice with AIA compared to healthy control mice.

Figure 3 Immunohistochemistry of the inflammatory infiltrate in the aortic PVAT of mice with CIA

(**a**) Anti-CD3 detection of T cells with hematoxylin counterstain in healthy control aortas from mice with CIA (*left image*) and from mice displaying T cell infiltrates evidenced by brown DAB staining (*right image*). Images are taken at 20X magnification. (**b**) Schematic representation of immunohistochemistry staining for $CD3^+$ T cells showing primary antibody, HRP-conjugated secondary antibody used for DAB-based antigen detection. Perivascular adipose tissue, PVAT; blood vessel, V; lumen, L; 1^o ab, primary antibody; 2^o ab, secondary antibody; HRP, horseradish peroxidase; DAB, 3,3'-diaminobenzidine.

Table 1 Recommended concentrations of 5-HT for measuring thoracic aortic constriction responses.

Final 5-HT concentration in myography bath (mM)
1×10^{-9}
3×10^{-9}
1×10^{-8}
3×10^{-8}
1×10^{-7}
3×10^{-7}
1×10^{-6}
3×10^{-6}
1×10^{-5}
3×10^{-5}

Table 2 Recommended tissue processing cycle for aortas.

Process	Reagent	Time (min)
Dehydration	70% (v/v) alcohol	90
Dehydration	90% (v/v) alcohol	90
Dehydration	100% (v/v) alcohol	60
Dehydration	100% (v/v) alcohol	60
Dehydration	100% (v/v) alcohol	60
Dehydration	100% (v/v) alcohol	60
Dehydration	100% (v/v) alcohol	60
Clearing	Xylene	90
Clearing	Xylene	120
Clearing	Xylene	120
Infiltration	Paraffin wax	120
Infiltration	Paraffin wax	90
Infiltration	Paraffin wax	90
Infiltration	Paraffin wax	60