Video Article

Subcutaneous Angiotensin II Infusion using Osmotic Pumps Induces Aortic Aneurysms in Mice

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Abstract

Osmotic pumps continuously deliver compounds at a constant rate into small animals. This article introduces a standard protocol used to induce aortic aneurysms via subcutaneous infusion of angiotensin II (AngII) from implanted osmotic pumps. This protocol includes calculation of AngII amount and dissolution, osmotic pump filling, implantation of osmotic pumps subcutaneously, observation after pump implantation, and harvest of aortas to visualize aortic aneurysms in mice. Subcutaneous infusion of AngII through osmotic pumps following this protocol is a reliable and reproducible technique to induce both abdominal and thoracic aortic aneurysms in mice. Infusion durations range from a few days to several months based on the purpose of the study. AngII 1,000 ng/kg/min is sufficient to provide maximal effects on abdominal aortic aneurysmal formation in male hypercholesterolemic mouse models such as apolipoprotein E deficient or low-density lipoprotein receptor deficient mice. Incidence of abdominal aortic aneurysms induced by AngII infusion via osmotic pumps is 5 - 10 times lower in female hypercholesterolemic mice and also lower in both genders of normocholesterolemic mice. In contrast, AngII-induced thoracic aortic aneurysms in mice are not hypercholesterolemia or gender-dependent. Importantly, multiple features of this mouse model recapitulate those of human aortic aneurysms.

Video Link

The video component of this article can be found at http://www.jove.com/video/53191/

Introduction

Aortic aneurysms exhibit permanent luminal expansion of the aorta that portends rupture and usually leads to death. This disease occurs in both abdominal and thoracic aortic regions, which are termed as abdominal aortic aneurysms (AAAs) and thoracic aortic aneurysms (TAAs), respectively. Due to an incomplete understanding of molecular mechanisms and pathophysiologic processes, there is no proven medical therapy that can prevent expansion or rupture of either type of aortic aneurysms. Since it is difficult to acquire patient samples and perform experiments in humans directly, research focusing on defining mechanisms of AAAs has been frequently extrapolated from animal models. A commonly used animal model is subcutaneous infusion of angiotensin II (AngII) into mice. Compared to other surgical approaches for inducing AAAs in mice, such as intra-aortic elastase perfusion or peri-aortic application of calcium chloride that require laparotomy[1,2], this method does not require entry into the body cavity and requires minimal surgical expertise[3,4].

Subcutaneous infusion of AngII through osmotic pumps to induce AAAs was initially reported in low density-lipoprotein (LDL) receptor -/- mice fed a saturated fat-enriched diet[3], and subsequently in apoE -/- mice fed a normal laboratory diet[4]. Many recent studies have also demonstrated that AngII induces AAAs in normolipidemic mice[5-7]. The approach of infusing AngII has been applied to induce AAAs and explore molecular mechanisms as well as development of potential therapeutic strategies (*e.g.*, [5-15]) since this model recapitulates many features observed in human AAAs. For example, risk factors of human AAAs such as smoking, aging, and male gender also augment AngII-induced AAAs in mice[16,17]. The association of hypercholesterolemia with AAAs in humans requires clarification. However, it has been consistent that hypercholesterolemia augments AngII-induced AAAs in mice[18]. Pathologies of AngII-induced AAAs in mice are highly heterogenous and are characterized by profound macrophage infiltration, collagen degradation, thrombotic formation and resolution, and neovascularization[19-21]. In contrast to the most common infrarenal aortic location of AAAs in humans, AngII-induced AAAs in mice occur in the suprarenal aortic region. Another ubiquitous feature of AngII-induced AAAs is the transmural medial break, leading to transmural thrombosis. It is unclear whether transmural elastin rupture occurs in humans since pathological development of AAAs in humans has not been exclusively studied due to lack of aneurysmal tissues from earlier stages.

AngII infusion into mice also leads to profound expansion of the thoracic aortic region, that is predominantly restricted to the ascending aorta which is the most common region for TAAs in humans[19,22-26]. Similar to AngII-induced AAAs, TAAs induced during AngII infusion also recapitulate many features of human TAAs[25]. However, in contrast to AngII-induced AAAs, AngII-induced TAAs are not associated with hypercholesterolemia and do not have gender differences.

The overall goal of subcutaneous AngII infusion into mice is to study pathological features and molecular mechanisms of AAAs and TAAs.

Protocol

Ethics Statement: Mouse studies are performed with approval of the University of Kentucky Institutional Animal Care and Use Committee (IACUC protocol number: 2006-0009). Mice are euthanized at termination using an overdose cocktail of ketamine (~ 210 mg/kg) and xylazine (~ 30 mg/kg).

1. Calculation of AngII Amount

NOTE: This protocol uses the example of infusion of AngII (1,000 ng/kg/min) for 4 weeks in 4 male LDL receptor -/- mice fed a saturated fat-enriched diet.

1. Weigh study mice before calculating the amount of AngII needed for infusion.
2. Use the template (**Table 1**) to calculate the AngII mass needed for the experiment. Use the “Mean Pumping Rate” indicated in the Instruction of pumps as the “Pumping rate” in Step 4 of the template. In the template, record Steps 1 - 5 manually, and Steps 6 - 10 are calculated automatically.
    1. In the template, assume that mice will gain 1 g of body weight during infusion of AngII 1,000 ng/kg/min for 4 weeks.
    NOTE: Each mouse may have very different body weight gain that will depend on many variables, such as mouse strain and diet. We routinely use “0" or “1 g” based on our own experience from previous studies.
    2. Calculate a 300 µl total volume of AngII solution for each mouse since each pump requires approximately 250 µl.

2. Dissolution of AngII

1. Store lyophilized AngII vials at -20 °C. Equilibrate AngII vials to RT before opening.
2. Weigh the calculated AngII mass (7.3 mg as shown in **Table 1**) into a sterile plastic tube.
NOTE: Per Merck Index, do not use glass tubes for the dissolution since an aqueous solution of AngII has a strong affinity for binding to glass.
3. Add the calculated volume of sterile saline (1,200 µl) into the plastic tube containing the lyophilized AngII, cap, and mix thoroughly by inversion until the solution is clear.
4. Label mouse numbers #1, #2, #3, and #4 on individual sterile plastic tubes with caps (0.5 - 1.5 ml). Prepare AngII solution under a laminar hood for each mouse based on body weight as calculated in Step 1.2 and **Table 1**.
    1. For example, pipette 3.6 µl sterile saline into tube #1, then 296.4 µl AngII solution, and mix thoroughly by pipetting up and down gently.
5. Label mouse numbers on plastic tubes with caps (4 ml; sterile). These will be used for incubating pumps as described in Step 3.13.

3. Osmotic Pump Filling

1. Obtain pumps in two separate parts: the main body of the pump and the flow moderator (**Figure 1**). Each box has 10 pump bodies and flow moderators that are wrapped individually. Record the lot number.
NOTE: Always wear gloves because oils transferred from hands to the exterior casing of pumps will adversely affect pumping function. Use sterile gloves, gauze, tubes, filling needle, and weigh boats to prepare the pumps, to avoid risk of infection from the implant.
2. Open only the number of pump bodies and flow moderators needed for the study, as these cannot be stored once opened. If more than 10 pumps are needed, ensure that the lot numbers of the pumps are the same for one study, since pumps from different lot numbers have different Mean Fill Volume and Pump Rate.
3. Weigh each pump (including both the main body and flow moderator), and note the weight to 4 decimal places (*e.g.*, 1.1443 g of mouse #1). This weight, termed “Pump Weight empty” in the template (**Table 1**), will be used to calculate the filled ratio.
4. Attach the pump filling needle to a 1 cc sterile syringe and carefully fill the syringe with AngII solution from the appropriately numbered plastic tube. It is important to avoid drawing air into the syringe.
5. Remove all air bubbles carefully from the syringe while the needle is positioned downwards. Keep the needle/syringe in this position to prevent the introduction of bubbles into the pump.
6. Gently insert the filling needle into the pump body. Advance the tip of the needle into the pump. Do not rest the tip of the needle tightly on the bottom of the pump.
7. Push the syringe plunger slowly to fill the pump with AngII solution. A dark shadow inside the pump indicates the filling level. The filling volume is approximately 246 µl, per Instructions.
8. Stop filling the pump and carefully remove the needle as soon as a bead of fluid rises out of the pump.
9. Insert flow moderator into pump through the hole on top of pump body until no gap is seen between the head of the flow moderator and the top of the pump body (**Figure 1**).
10. Insertion of moderator into the pump body leads to some fluid leaking from the opening of the flow moderator. Carefully blot all extra fluid that might have leaked during placement of moderators.
11. Weigh filled pump. Record the weight under “Pump Weight Filled” in the template.
12. Calculate Filling Ratio (%) = (Pump Weight “filled” - “empty”) x 1,000/mean fill volume x 100.
    1. Calculate Filling Ratio as indicated in Table 1. Ideally, filling ratio should be equal or greater than 100%. Refill pump if the filling ratio is < 95% (implicating that air bubbles may be present in pump).
13. Place filled pump into the labeled 4 ml tube (Step 2.5) with the moderator head facing upward. Add sufficient volume of sterile saline to cover the pump. Keep pump in tube of saline until implantation.

14. Place tubes in a 37 ºC incubator. Incubate pumps O/N (at least 12 hr) to allow partial priming, and then implant into mice. Pumping of AngII starts approximately 24 hr after implantation, which allows mice to recover from surgery prior to any potential stress arising during AngII infusion.

4. Preparation for Pump Implantation

1. Autoclave (gravity mode, dry cycle, 15 min) gauze, cotton swabs and surgical tools including scissors, hemostat, forceps, staples, and stapler at least 1 day prior to surgery.
2. In a procedure room, prepare vaporizer for anesthesia using isoflurane. Open up sterile drapes in a laminar flow hood, and place nose cone for isoflurane anesthesia. Place betadine, 70% ethanol, sterile water, bead sterilizer, antiseptic handrub, swabs, gauze, and filled pumps in a laminar hood.
3. Don a mask and gown, then open an outer drape in a laminar hood with clean hands. Put on sterile gloves and open the sterile inside pack.

5. Surgical Procedure of Pump Implantation

1. Place mouse in an induction chamber with inflow of isoflurane at a flow rate of 1.5 - 2%. Monitor the mouse for an additional 2 - 3 min after recumbency. Shave an area about the size of a quarter, over the left or right shoulder.
2. Place mouse in a laminar hood, with its nose flush with a cone connected to isoflurane outflow (**Figure 2A**). Place the mouse's head toward the surgeon's dominant hand. Use vet ointment on mouse's eyes to prevent dryness while under anesthesia. Ensure that the mouse has no response to stimulation of pain prior to the surgery. For example, pedal response is a good indicator for pain.
3. Swab and wipe shaved area with betadine followed by three wipes with 70% ethanol. Don or change sterile gloves.
4. Use a surgical scalpel to make an ~ 1 cm incision behind the ear over the shoulder blade of the front leg. This incision should be perpendicular to the tail. Use care to cut only the skin and not underlying tissues.
5. Hold forceps in one hand to open incision, and use the other hand to make a subcutaneous tunnel under the skin using a hemostat (**Figure 2B**).
6. Advance hemostat tip toward the tail, and create a pocket for pump. This is accomplished by carefully opening the jaws of the hemostat under the skin to open up a pouch. Withdraw the hemostat from incision.
7. Insert pump into the incision with the moderator head positioned to the rear of the mouse (**Figure 2C**). Gently push the pump completely into the pocket. There should be enough free space to close the wound with no tension or stretching of the skin.
8. Once a pump has been inserted, firmly pinch both sides of the incision, straightening so the edges meet, and place 1 or 2 wound clips to close (**Figure 2D**). Inspect the incision site to ensure that there is complete closure of the wound, and that the pump is not pressing directly on the site.
9. Apply topical lidocaine cream (4% wt/wt) with a clean cotton swab.Remove mouse from the nose cone, and place it on a heating pad until it regains consciousness. After recovering, the mouse is returned to its cage.
10. Place surgical instruments into a bead sterilizer for 10 seconds between mice. Allow instruments to cool before use. Clean gloves with antiseptic handrub between mice. Monitor all mice until full recovery is achieved.
11. Monitor mice closely after the surgery. Inject a bolus of sterile saline (0.2 - 0.3 ml) subcutaneously if a mouse shows signs of distress, dehydration or apparent weight loss. Observe mice at least twice a day during the first 10 days, and at least once every day subsequently. Perform a necropsy immediately if any mice die during AngII infusion. Remove wound clips between 7 - 14 days after the surgery.

6. Harvesting, Fixing, Cleaning, and Imaging of Aortas

1. Cut open the mouse thoracic and abdominal cavities ventrally, cut open right atrium, perfuse with saline through left ventricle of the heart to remove blood in the aorta, and then harvest the aorta [27].
2. Place harvested aortas in plastic tubes containing at least 3 ml of 4% paraformaldehyde or 10% neutrally buffered formalin for 24 - 48 hr [27].
3. Remove adventitial tissues carefully. Pin aorta on black wax with pins. Acquire aortic images with same magnification. Include a ruler in each image for calibration, as shown in **Figure 3**.

7. En face Imaging of Aortas

1. Cut open aorta longitudinally through the outer and inner curvature of aortic arch, and cut open major branches including innominate, left carotid artery, and left subclavian artery. Pin aorta flat with outer adventitial laying adjacent to the black wax.
2. Acquire en face picture of the intimal surface of aorta at the same magnification. Include a ruler in each image for calibration, as shown in **Figure 4**.

Representative Results

The 4 male LDL receptor -/- mice described in the Protocol section were euthanized after 4 weeks of AngII infusion. Aortas were harvested, cleaned, and imaged to visualize aortic dilations. As shown in **Figure 3**, aortas have several different characteristics including expansion of the suprarenal region (AAAs; **Figure 3A**), expansion of the ascending region (TAAs; **Figure 3B**), or expansion of both regions (presence of both AAAs and TAAs; **Figure 3C**), whereas morphology in one mouse was grossly normal (**Figure 3D**). Dilation of the abdominal aorta is quantified by measuring the *ex vivo* maximal width of the suprarenal region, as illustrated by the red line in **Figure 3A**. To measure ascending aortic dilation, aortas were cut open and pinned as shown in **Figure 4**. Intimal surface area was measured in the ascending aortic region (area surrounded by the red lines in **Figure 4A**) to quantitate TAAs. A ruler was included in each image to standardize measurements, as shown in both **Figures 3 and 4**.

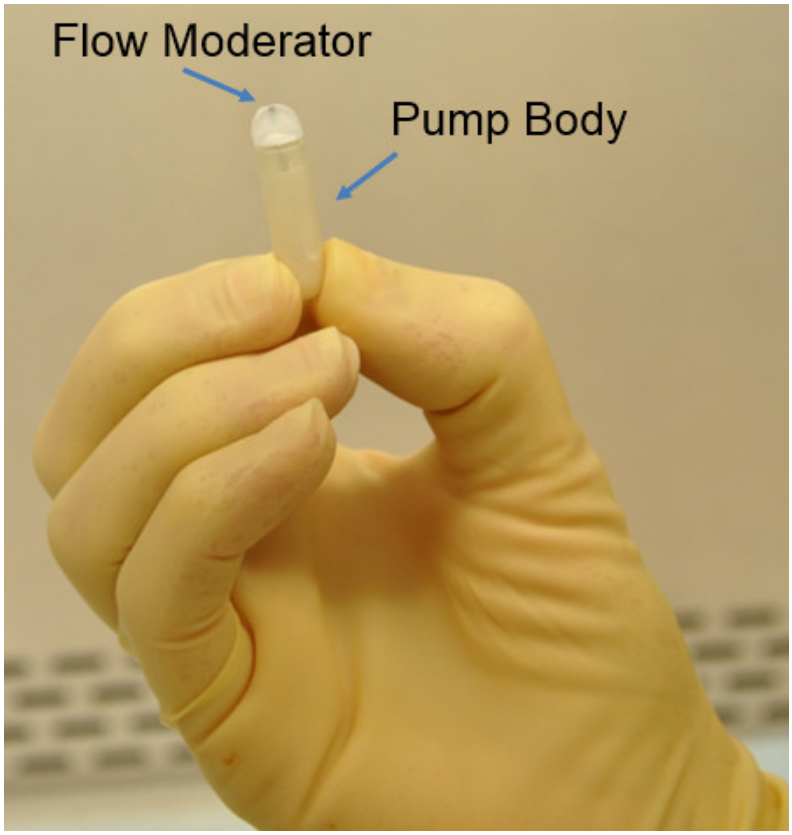

Figure 1. Representative image of filled osmotic pump. Each pump contains two separate parts: a main body and a flow moderator. After filling the pump body with AngII, the flow moderator is inserted to seal the pump.

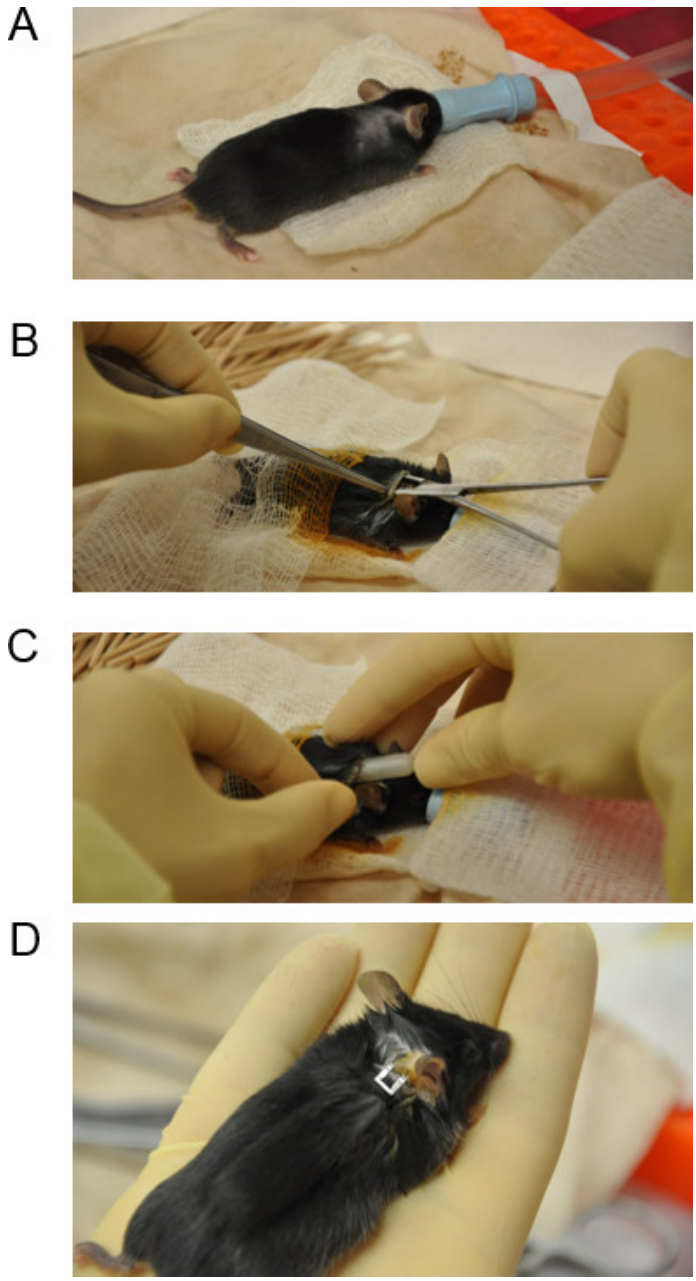

Figure 2. Process of pump implantation surgery. (**A**) Mouse is placed in a laminar hood with a nose cone that is continuously releasing isoflurane and oxygen; (**B**) A straight hemostat is inserted into the skin incision to make a subcutaneous tunnel; (**C**) Pump is inserted through the skin incision gently; (**D**) The skin incision is stapled after pump insertion.

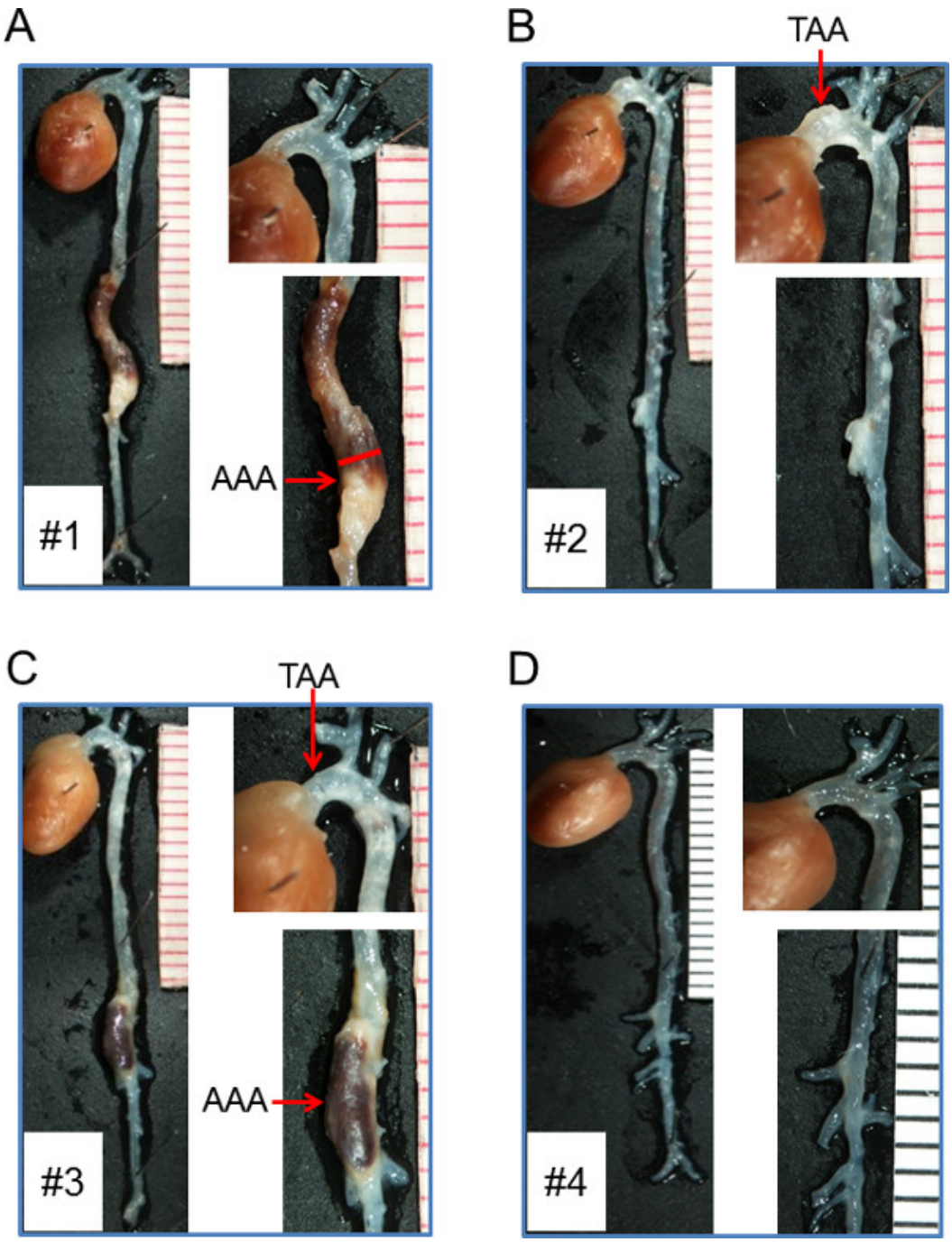

Figure 3. Aortic images (*ex vivo*) from mice infused with AngII. AngII 1,000 ng/kg/min was infused in male LDL receptor -/- mice for 28 days. (**A**) AAAs accompanied by thrombosis; Red line (2.05 mm) shows the measurement of maximal aortic width in the suprarenal region. (**B**) Ascending aortic dilation (TAA) with grossly normal abdominal aorta; (**C**) Profound dilations in both the ascending and suprarenal aortic regions (TAAs and AAAs); (**D**) grossly normal aorta with no apparent dilation of either ascending or suprarenal aortic region.

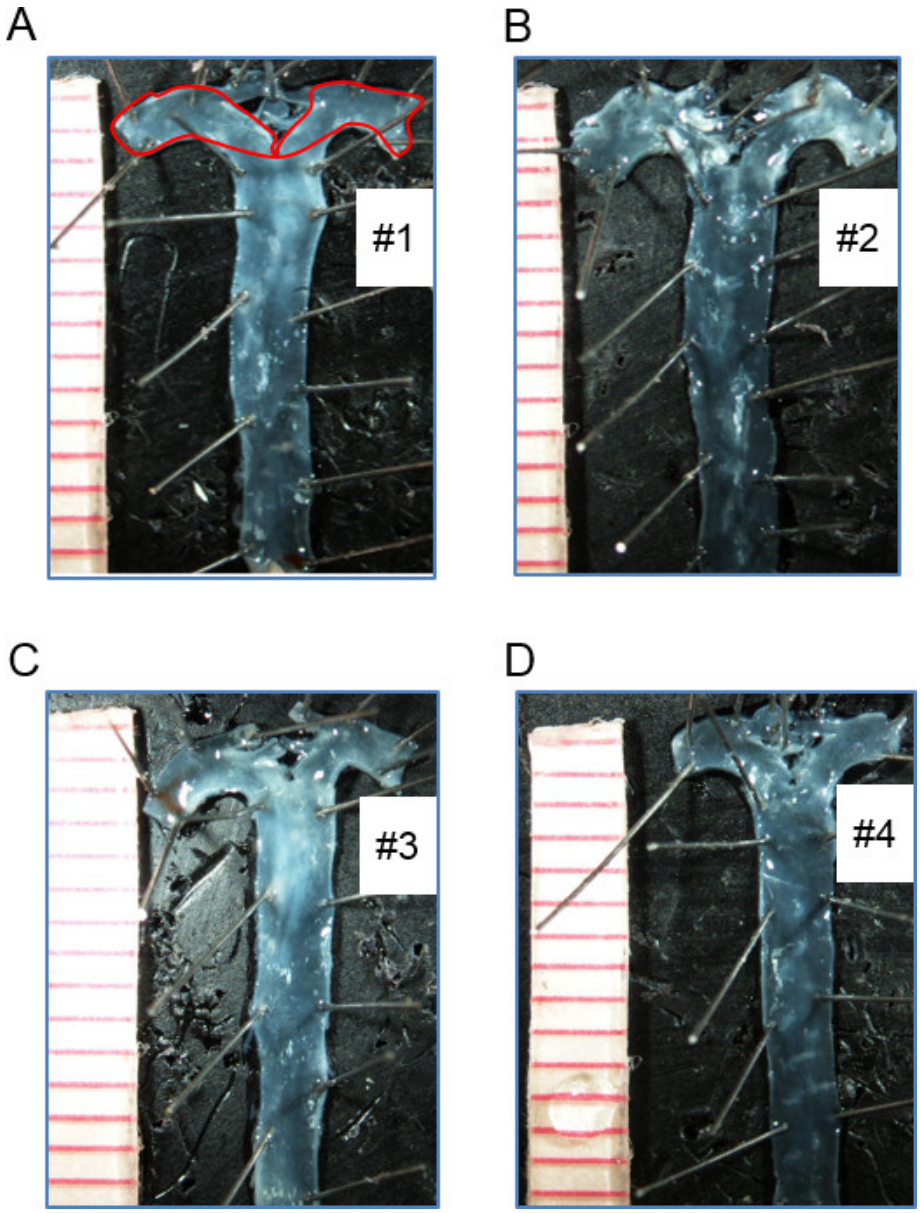

Figure 4. En face images of thoracic aortic regions from mice infused with AngII. AngII 1,000 ng/kg/min was infused in male LDL receptor -/- mice for 28 days. Surface area outlined by a red line represents the ascending aortic region including part of the aortic arch.

1	Dose required			1,000	ng/kg/min		
2	Start body weight (largest mouse)			24.8	g		
3	Total estimated body weight gain			1	g		
4	Pumping rate			0.25	µl/hr		
5	Number of mice			4			
6	Dose per hour for animal			1518	ng		
7	Conc needed			6072	ng/µl		
8	For 300 ul solution			1.82	mg/300 µl		
	SOLUTION NEEDED						
9	Total AngII (mg)			7.3	mg		
10	Dissolved in saline			1,200	µl		
Mouse	Body Weight	Dilution factor	Volume (µl)		Pump Weight (g)		Filled Ratio

#	(g)		AngII	Saline	Empty	Filled	(%)
1	24.5	1.0	296.4	3.6	1.1443	1.3877	99
2	23.0	0.9	278.2	21.8	1.1677	1.4145	100
3	24.8	1.0	300.0	0.0	1.1438	1.3904	100
4	21.8	0.9	263.7	36.3	1.1438	1.3904	100
Dilution factor = body weight of the mouse/body weight of the largest mouse							

Mouse	Body Weight	Dilution factor	Volume (µl)		Pump Weight (g)		Filled Ratio
#	(g)		AngII	Saline	Empty	Filled	(%)
1	24.5	1.0	296.4	3.6	1.1443	1.3877	99
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3	24.8	1.0	300.0	0.0	1.1438	1.3904	100
4	21.8	0.9	263.7	36.3	1.1438	1.3904	100

Table 1: Calculation for 28-day infusion via osmotic pumps.

Discussion

Osmotic pumps delivering AngII subcutaneously is a routine approach to inducing aortic aneurysms in mice. Based on data from many laboratories, there have been consistent findings that this is a reliable and reproducible method to study both AAAs[3,4] and TAAs[22-26] in mice. Therefore, this mouse model is considered a model that recapitulates several features of human aortic aneurysms and provides mechanistic insights into these devastating diseases.

While aging is a risk factor for AAAs in humans, it has not been systematically studied for AngII-induced AAAs in mice. However, it appears incidence and severity of AngII-induced AAAs are similar in mice at the age of 8 - 48 weeks[4,5,7]. Currently, there are only a few studies reporting AngII-induced TAAs in mice at the age of 8 - 24 weeks[22-26], which did not show apparent age-related differences on TAA formation.

Female mice have a much lower incidence of AAAs than male mice infused with AngII[4,28]. It is also worth noting that the incidence of AngII-induced AAAs is much higher in hyper- than normo-cholesterolemic mice, which is more than 50% versus less than 30%, respectively. Additionally, aortic rupture is frequent (approximately 10 - 30%) in both normo- and hypercholesterolemic mice during AngII infusion. Infusion of AngII at a rate of 1,000 ng/kg/min into hypercholesterolemic mice, such as LDL receptor -/- mice fed a Western diet or apolipoprotein (apoE) -/- mice fed a normal or Western diet, has maximal effects on AAA development[3,4,29]. This infusion rate is optimal for a study in which manipulating a gene of interest in hypercholesterolemic mice is expected to reduce AAAs. If a manipulation in hypercholesterolemic mice is expected to augment AAAs, it is recommended to infuse AngII at a rate of 500 ng/kg/min or lower[30]. In contrast to AAAs, there is no demonstrated association between male gender or hypercholesterolemia and AngII-induced TAAs[25]. However, similarly to AAAs, if manipulation of a gene of interest is expected to augment TAAs, we recommend a lower infusion rate than 1,000 ng/kg/min for AngII infusion.

It is also important to know that incidence and severity of AngII-induced aortic aneurysms vary among individual mice and between studies. If mice do not develop aortic aneurysms, one potential possibility is that AngII might have not been successfully delivered into mice. For validation of a high infusion rate of AngII, such as 1,000 ng/kg/min, measurement of blood pressure is recommended prior to, and during, AngII infusion using a non-invasive tail-cuff method[31]. AngII infusion at a rate of 1,000 ng/kg/min increases systolic blood pressure in mice. Also, plasma renin concentrations may be measured during AngII infusion or at termination since AngII has a negative feedback on renin secretion. Therefore, AngII infusion leads to reductions in plasma renin concentrations. If a mouse infused with AngII has no apparent aortic pathologies, no increase of blood pressure, and no decrease of plasma renin concentration, it would indicate that AngII has not been delivered efficiently through the implanted osmotic mini pump. We would recommend removing this mouse from the study. It is also important to note that some mice do not develop aortic aneurysms despite increased systolic blood pressures and decreased plasma renin concentrations. These mice should remain in the study.

In summary, AngII infusion is achieved by subcutaneous implantation using osmotic pumps to induce aortic aneurysms in mice. This method delivers AngII constantly at a defined rate for designated durations that are used to study both AAAs and TAAs.

Disclosures

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