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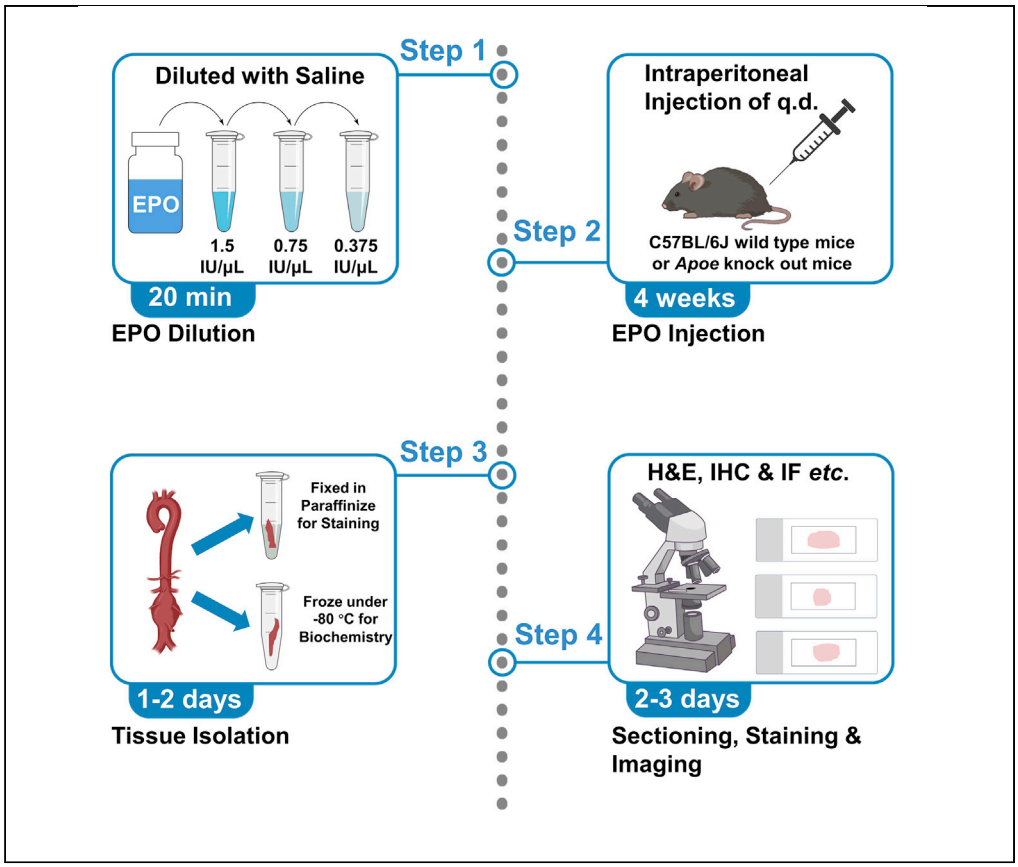
Protocol

An animal model of EPO-induced abdominal aortic aneurysm in WT and *Apoe*$^{-/-}$ mice



Abdominal aortic aneurysm (AAA) is a potentially fatal vascular disease, but the underlying mechanisms remain obscure. Here, we provide a protocol using erythropoietin (EPO) to induce the formation of AAA in both wild-type (WT) and apolipoprotein E (*Apoe*$^{-/-}$) mice. We describe the dose, manner, and timing of EPO administration. We also detail mice dissection, aorta isolation, and histological analysis. The animal model of EPO-induced AAA provides a useful tool for exploring the mechanism of AAA in experimental studies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for EPO-induced AAA in WT and *Apoe*$^{-/-}$ mice

Steps for preparation and intraperitoneal injection of EPO into mice

Detailed description for isolation, section preparation, and staining of aorta

Useful for modeling the pathological characteristics of human AAA

Zhang et al., STAR Protocols 4, 101929
March 17, 2023 © 2022 The Author(s).
https://doi.org/10.1016/j.xpro.2022.101929

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An animal model of EPO-induced abdominal aortic aneurysm in WT and *Apoe*$^{-/-}$ mice

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https://doi.org/10.1016/j.xpro.2022.101929

SUMMARY

Abdominal aortic aneurysm (AAA) is a potentially fatal vascular disease, but the underlying mechanisms remain obscure. Here, we provide a protocol using erythropoietin (EPO) to induce the formation of AAA in both wild-type (WT) and apolipoprotein E (*Apoe*$^{-/-}$) mice. We describe the dose, manner, and timing of EPO administration. We also detail mice dissection, aorta isolation, and histological analysis. The animal model of EPO-induced AAA provides a useful tool for exploring the mechanism of AAA in experimental studies.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2021).[1]

BEFORE YOU BEGIN

Abdominal aortic aneurysm (AAA) is a potentially fatal vascular disease, but the underlying mechanisms remain obscure. We describe a useful mouse model of AAA induced by erythropoietin (EPO) injection. The pathological characteristics of AAA mouse model induced by EPO are closer to those of human AAA than the traditional AAA mouse model induced by angiotensin II (AngII) infusion. Because severe hyperlipidemia (apolipoprotein E knockout, *Apoe*$^{-/-}$) is necessary to achieve a high incidence of AAA in the AngII-induced mouse model. EPO injection leads to a dose-dependent increase in mortality, AAA incidence and abdominal aortic diameters of wild-type (WT) and *Apoe*$^{-/-}$ mice. This protocol below describes how EPO promotes the formation of AAA in WT mice as well as in *Apoe*$^{-/-}$ mice.[1]

Institutional permissions

We have obtained the approval of all animal experimental procedures from the Ethics Committee on Animal Experiments of Shandong University Qilu Hospital and perform in accordance with the Animal Management Rules of the Chinese Ministry of Health. Approval number: DWLL-2014-024.

Animal preparation

Timing: 1 week

1. Purchase eight-week-old male C57BL/6J (WT) or *Apoe*$^{-/-}$ mice from Animal Experimental Center.

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Note: There is no significant difference in mortality and incidence of AAA between WT and *Apoe*$^{-/-}$ mice following this protocol,[1] so that readers can choose either one according to their own experiments. *P* value for incidence of AAA in *Apoe*$^{-/-}$ vs WT mice receiving high-dose EPO is 1.000.

Note: Use PROC POWER procedure in the software SAS to estimate the sample size. Set the significance threshold (α) and power (1 − β) at 0.05 and 80%, respectively. Effect sizes between groups are based on preliminary experiments and related published papers.[2,3] Accordingly, mouse number in each experimental group should be no less than 15.

Note: You can also choose female mice for experiments. In our study, the incidence of AAA in male and female WT mice receiving medium-dose EPO is 53.3% and 33.3% respectively ($P = 0.462$).[1]

2. Randomly divide all mice into 4 groups for treatment: the vehicle group, and the low-, medium- and high-dose of EPO groups.
3. Let all mice to adapt environment for one week. Keep all mice on a 12-h light/12-h dark cycle in specific pathogen free conditions with food and water freely available.

Note: Feed WT mice with a normal diet. Feed *Apoe*$^{-/-}$ mice with a high-fat diet or a normal diet, which does not affect the incidence of AAA or the mortality. In our study, the incidence of AAA in *Apoe*$^{-/-}$ mice (high-dose EPO) fed with a high-fat diet or a normal diet is 60% and 60% respectively, and the mortality in *Apoe*$^{-/-}$ mice (high-dose EPO) fed with a high-fat diet or a normal diet is 33.3% and 26.7% respectively ($P = 1.000$).[1]

4. Measure the body weight of each mouse every week.

EPO dilution

Timing: 20 min

5. Purchase recombinant human EPO from Shenyang 3SBIOINC, China (Concentration: 10,000 IU/mL).

Note: The identities of amino acid sequence of EPO between human and mouse are 80%, and the recombinant human EPO could significantly increase the number of red blood cell in mice,[1] so we choose the clinical-used EPO injections. EPO from other source only if with biological activity might also be available.

6. Define the EPO dose in the low-, medium- and high-dose groups as 2,500 IU/kg (body weight) /day, 5,000 IU/kg/day and 10,000 IU/kg/day, respectively.

Note: The low dose of EPO used in mice refers to a previous study where 3,000 IU/Kg of EPO, three times a week for 8 weeks.[4] We find that the incidence of AAA is EPO dose-dependently increased in mice and only the high dose of EPO (10,000IU/kg/day) results in an incidence of AAA similar to that induced by AngII treatment at a typically high dose (1.44 mg/kg/d).

7. Dilute EPO solution to 1.5 IU/μL in high-dose group, 0.75 IU/μL in medium-dose group, and 0.375 IU/μL in low-dose group, respectively, with sterile normal saline.

Note: Prepare the EPO solution under a sterile and dark condition in a biosafety cabinet. Troubleshooting 1.

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Note: Store the diluted EPO solution at 4°C in a dark place and used in 48 h. We did not test the diluted EPO solution for longer storage.

8. Calculate the total solution volume for intraperitoneal injection in each mouse as: $\frac{body\ weight\ (g)}{30\ (g)} \times 0.2\ mL$.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Recombinant human erythropoietin injection (CHO cell)	Shenyang 3SBIOINC, China	Concentration: 10,000 IU/mL
Normal saline	KELUN PHARMACEUTICAL Co., Ltd. China; Alternative choice: Merck, S8776	N/A
Sodium pentobarbital	Sinopharm Chemical Reagent Co., Ltd. China; Alternative choice: Merck, Y0002194	69020181
4% paraformaldehyde	KeyGEN BioTECH Co., Ltd. China; 4% Alternative choice: Merck, 1004965000	KGIHC016CS
Histo-Clear	Wuxi Jiangyuan Industrial Technology and Trade Corporation, China; Alternative choice: HistoChoice® Clearing Agent (H2779) or Xylenes (214736) from Sigma-Aldrich	N/A
Ethanol	Sinopharm Chemical Reagent Co., Ltd. China; Alternative choice: Merck, 493538	N/A
Paraffin	Leica	39601006
Hematoxylin staining solution	Servicebio Biotechnology Co., Ltd.	G1005-1
Eosin staining solution	Servicebio Biotechnology Co., Ltd.	G1005-2
Neutral balsam	Solarbio Co., Ltd.	G8590
Critical commercial assays		
Elastic (Connective Tissue Stain) Kit	Abcam	ab150667
Experimental models: Organisms/strains		
WT mouse	Beijing Huafukang Animal Experimental Center	C57BL/6J
Apoe$^{-/-}$ mouse	Jackson Laboratory	002052
Other		
Biosafety cabinet	Thermo Fisher Scientific	1379
Pipette of 1,000 μL	Eppendorf	Research plus (100–1,000 μL)
Pipette of 200 μL	Eppendorf	Research plus (20–200 μL)
Pipette of 100 μL	Eppendorf	Research plus (10–100 μL)
Vernier caliper	Mitutoyo Co., Ltd.	1204-70
4°C freezer	HEFEI MEILING Co., Ltd.	BCD-201KCK
−80°C freezer	New Brunswick Scientific	Innova U725
Microscopic scissors	Hangzhou 66vision Medical Apparatus Co., Ltd. Alternative choice: Wise Linkers, 4336853602	54109B
Microscopic tweezers	SHINVA MEDICAL INSTRUMENT Co., Ltd. Alternative choice: Profix, 15JP	ZD270RN
Single-use sterile syringe (1 mL, 2 mL, 20 mL)	Shandong Weigao Group Medical Polymer Co., Ltd. China; Alternative choice: HENKE-JECT, 40127200V0, 4022L000V0, 4202H000V0	N/A
Tissue freezing tubes	Corning	430659
Aluminum foil	TOP Daily Chemicals (China) Co., Ltd.	MF26

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Benchtop centrifuge	Heraeus	labofuge 400
Adhesion microscope slides	Citotest Labware Manufacturing Co., Ltd.	188105
Tissue embedding cassettes	Citotest Labware Manufacturing Co., Ltd.	31050102W
Coverslip	Citotest Labware Manufacturing Co., Ltd.	10212450C
Liquid nitrogen tanks	ChartIndustry (Chengdu) Company Co., Ltd.	YDS-120-216
Ice making machine	Scotsman	AF-156
Automatic Benchtop Tissue Processor	Leica	TP1020
Heated Paraffin Embedding Station	Leica	HistoCore Arcadia H
Cold Plate	Leica	HistoCore Arcadia C
Rotary Microtome	Leica	RM2245
Water bath for paraffin sections	Leica	HI1210
Flattening table for histopathology	Leica	HI1220
Multifunctional Slide Stainer	Leica	ST5020
Fully Automated Glass Coverslipper	Leica	CV5030
Upright Microscope	Olympus Corporation	BX41-12P05
Stereo Microscope	Olympus Corporation	SZ51
Normal diet	Xietong Pharmaceutical & Biological Engineering Co., Ltd. China; Alternative choice: Research Diets, Inc., D10012M	SWS9102
High-fat diet	Trophic Animal Feed High-tech Co., Ltd. China; Alternative choice: Clinton-Cybulsky diet, Research Diets, Inc. D12108C	TP28521
Procoagulant tubes	Becton, Dickinson and company	367955
EDTA-containing anticoagulation tube	Becton, Dickinson and company	367856

MATERIALS AND EQUIPMENT

Alternatives: Recombinant human erythropoietin can also be purchased from R&D Systems (catalog #: 287-TC-500).

STEP-BY-STEP METHOD DETAILS

EPO injection

Timing: 4 weeks

This section describes the induction of AAA formation in three groups of WT mice receiving an intraperitoneal injection of EPO in a dose of 2500, 5000, and 10,000 IU/kg per day, respectively, for 4 weeks. Moreover, this protocol is also suitable for *Apoe*$^{-/-}$ mice.

1. Calculate the injection volume for each mouse based on grouping and body weight. Give the control group normal saline via intraperitoneal injection.

⚠ CRITICAL: Perform EPO injection in a dark place. Troubleshooting 1.

Note: Carried out the EPO intervention at the same time every day for four weeks.

Note: The manner of EPO administration in this protocol is intraperitoneal injection, which is fast in drug absorption, easy in operation and steady in repeatability. However, intravenous injection is difficult to operate every day via vein in mouse. The absorption rate of drug via

Figure 1. Inject EPO solution into the peritoneal cavity of mice

subcutaneous administration is slower than that of intraperitoneal injection. So that the intraperitoneal injection may be the best choice for both EPO absorption and easy operation.

2. Grabbing mice.
   a. Grasp the fur on the mouse's hackles and secure the head to prevent a bite.

   Note: Use moderate force to prevent the mouse from suffocating.

   b. Fix the tail of the mouse with the ring finger or little thumb of the left hand, so that the whole trunk of the mouse can be fixed and kept upright (Figure 1).

3. Injection.
   a. After grasping the mouse, try to keep the mouse abdomen in a level higher than the mouse head.

   Note: Ensure the abdominal organs moving to a lower position to avoid stabbing injuries.

   b. Hold the 1 mL syringe in the right hand, and insert the needle slowly into the mouse abdomen at 30–45 degrees at a depth of less than 1 cm with a sensation of the disappearance of needle resistance and injection into a cavity.
   c. Draw back the syringe to make sure the presence of a negative pressure and then inject the solution into the abdomen.
4. Needle removal: To prevent leakage, rotate the needle slightly and then slowly pull it out.

   Note: If you see a bulge on the skin, it is possible that you have made a subcutaneous injection.

5. Monitor the mice daily for diet intake, water drinking, behavior, health status and deaths, during EPO injection. Approximately 15 days after the first injection, mice in the high-dose EPO group begin to develop AAA.

   Note: With the injection of EPO, the skin of mice may turn red, but the mobility of mice remains normal.

   Note: If you find a mouse manifested with bilateral lower limbs paralysis, it is likely that a dissecting AAA may have developed and this mouse is at a high risk of death (Figures 2A and 2E). In this case, you have to end the EPO intervention, euthanize, and dissect the mouse to check the aorta (Figures 2B and 2F).

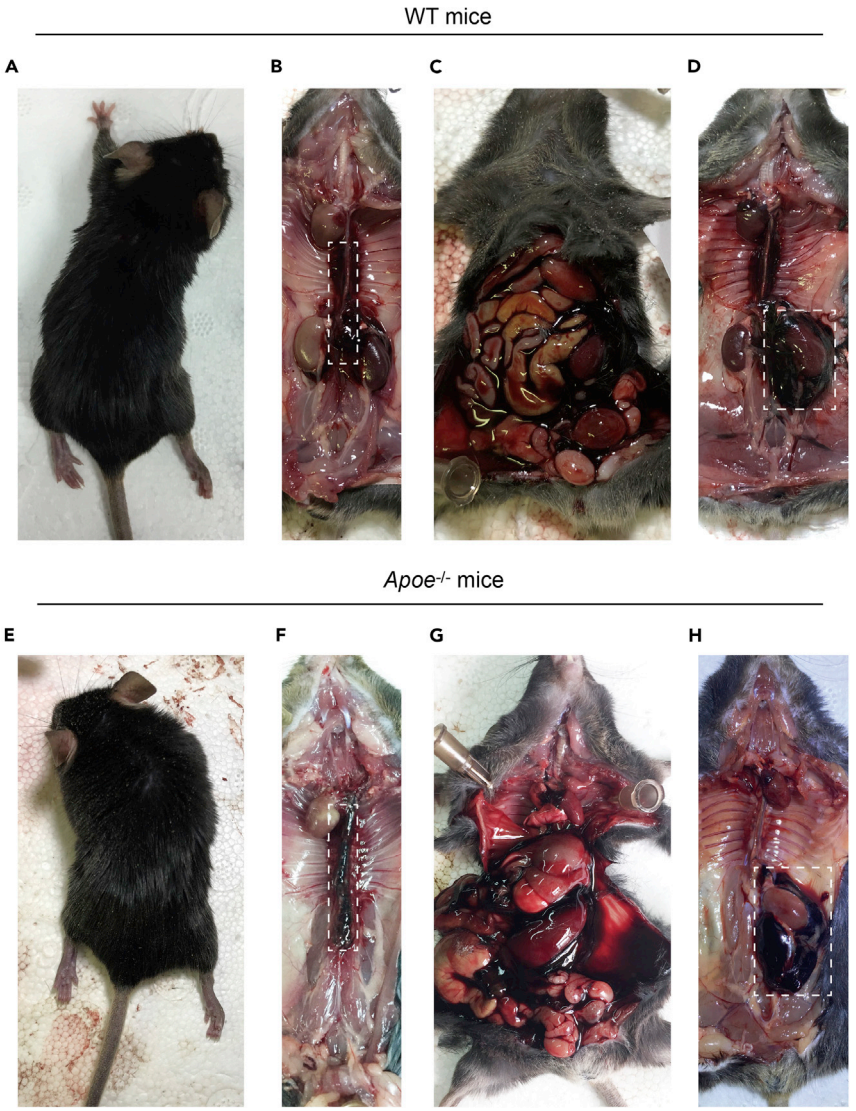

Figure 2. Representative photographs of WT and *Apoe*$^{-/-}$ mice with a dissecting AAA after EPO injection

(A) A WT mouse had bilateral lower limbs paralysis.
(B) The mouse (A) developed a dissecting AAA (indicated by the dashed frame).
(C) A WT mouse developed massive intra-abdominal hemorrhage.
(D) A WT mouse suffered from a large abdominal aortic hematoma (indicated by the dashed frame).
(E) An *Apoe*$^{-/-}$ mouse had bilateral lower limbs paralysis.
(F) The mouse (E) developed a dissecting AAA (indicated by the dashed frame).
(G) An *Apoe*$^{-/-}$ mouse developed massive intra-abdominal hemorrhage.
(H) An *Apoe*$^{-/-}$ mouse suffered from a large abdominal aortic hematoma (indicated by the dashed frame).

6. In the case of sudden death of a mouse, perform an autopsy to ascertain the cause of death. You may see a massive intra-abdominal hemorrhage (Figures 2C and 2G) or a large abdominal aortic hematoma (Figures 2D and 2H). You may observe thoracic hemorrhage occasionally.

Euthanasia and tissue isolation

Timing: 1–2 days

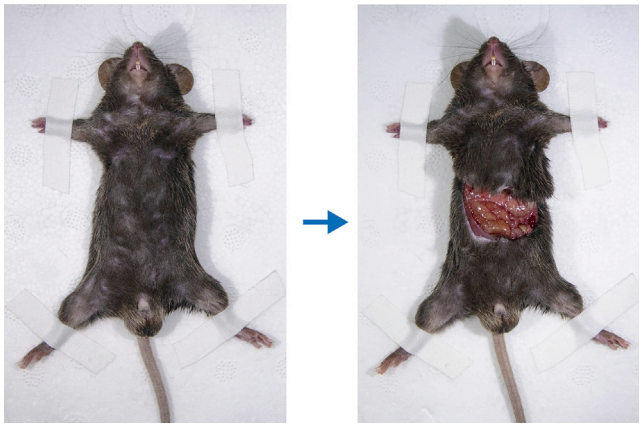

Figure 3. Fix the mice on the bench, cut the abdominal skin with a U-shape incision, and open the abdominal cavity

This section describes the details of euthanasia and aorta isolation.

7. Anesthetize the mice with intraperitoneal injection of sodium pentobarbital (80 mg/kg), and then euthanize the mice by cervical dislocation after deep reflex has disappeared.

   Note: The process of euthanasia must be in accordance with recommendations of international guidelines.

8. Fix the mice on the bench, cut the abdominal skin with a U-shape incision, and open the abdominal cavity (Figure 3).

   ⚠ CRITICAL: Be careful not to damage the liver when cutting the skin and peritoneum.

9. Cut the diaphragm along the edge of the ribs and bluntly isolate the fatty tissue around the heart and the pericardium to clearly expose the heart.
10. Use a sterile syringe (1 mL or 2 mL) to draw about 1.5 mL of blood from the apical part of the heart. Troubleshooting 2.
11. Drop a small amount of blood into an EDTA-containing anticoagulation tube (Becton, Dickinson and company, 367856) and flick the wall to mix blood well for whole blood testing.

    Note: If needed, you can use the whole blood to measure red blood cells counting, hemoglobin concentrations, hematocrit values, white blood cell counting, monocyte counting, lymphocyte counting, granulocyte counting and platelet counting.

12. Place the remaining blood in a procoagulant tube (Becton, Dickinson and company, 367955) at 25°C for 30 min, and then centrifuge it at 900 *g* for 15 min.

    Note: If needed, you can use the collected serum to measure serum total cholesterol, low-density lipoprotein cholesterol, triglycerides, high-density lipoprotein cholesterol, alanine transaminase, aspartic transaminase, creatinine, blood urea nitrogen and serum EPO concentration.

13. Collect the serum and store it at −80°C in the freezer.
14. Cut and open the right auricle and perform *in vivo* perfusion using normal saline (approximately 20 mL) until the liver, kidney, fat, and other tissues are adequately perfused with clear fluid flowing out of the right auricle.

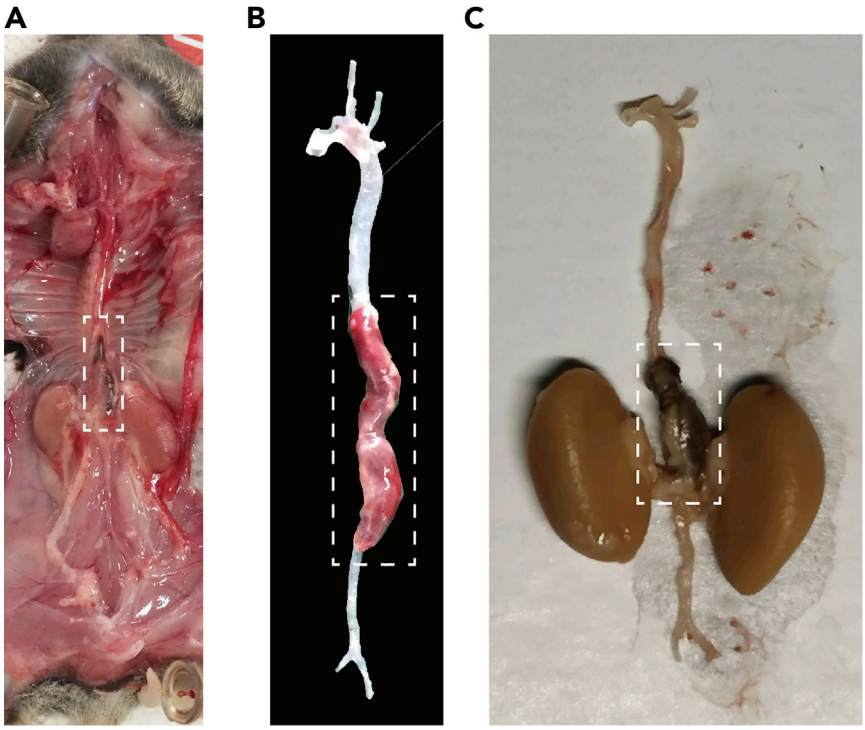

Figure 4. Representative photographs of WT mice after EPO injection

(A) A representative photograph of EPO-induced AAA *in vivo*.

(B) A representative photograph of isolated aorta with EPO-induced AAA.

(C) The relation between EPO-induced AAA and renal arteries in a mouse. The dashed frame indicated the AAA in the aorta.

15. Isolate the heart, liver, spleen, kidney, and aorta.

⚠ CRITICAL: Carefully separate the periaortic connective tissue (Methods video S1).

Note: Prepare the isolated liver and kidney into paraffin sections. Stained the sections with H&E to observe whether there are morphological changes in the liver and kidney tissue after EPO injection. Measure the weights of heart and spleen to assess the impact of EPO injections. If *Apoe*$^{-/-}$ mice were chosen in this experiment, you can evaluate the plaque area in the cross sections of the aortic root by H&E and oil red O staining.

Note: You can use a stereo microscope when separating the periaortic connective tissue. Soak the vascular tissue in cold normal saline to prevent degradation and complete the operation as soon as possible.

Note: Isolate and preserve the entire aorta from the ascending aorta to the iliac artery, preferably preserving the right and left common carotid arteries and the left subclavian artery (Figures 4A and 4B).

Note: The majority of EPO-induced AAA are proximal to the renal arteries (Figure 4C).

16. Measure the external diameters of the abdominal aorta using vernier caliper and take photographs of the entire aorta.

Note: Measure the maximum external diameter of the abdominal aorta with a vernier caliper. The definition of AAA is the localized dilation of the abdominal aorta with an external diameter exceeding the normal aortic diameter by 50%.[5]

17. Rapidly froze a part of the tissues in liquid nitrogen and then transfer them to −80°C freezer for storage. Fix the rest part of the tissues in 4% paraformaldehyde for 48 h for subsequent experiments.

Pathological testing

Timing: 2–3 days

This section describes the steps of AAA section preparation and staining.

18. Paraffin section preparation.
    a. Cut a section with a length of about 0.5 cm from the AAA segment with the largest external diameter and put it into a tissue embedding cassette.

Note: Label mouse groups on the cassettes with a pen that could not be removed in the aqueous or organic solvents, for example, a pencil.

Note: If the tissue sample is too small, carefully wrap the sample with a gauze to prevent tissue loss (Figure 5A).

    b. Immerse the cassettes in tap water, and rinse them with running water for 3–4 h to remove paraformaldehyde.
    c. Place the cassettes in an automatic benchtop tissue processor (Figure 5B) and select an appropriate program as follows:
        i., water for 10 min.
        ii., 70% ethanol for 2 h.
        iii., 80% ethanol for 2 h.
        iv., 90% ethanol for 2 h.
        v., 95% ethanol I for 1 h.
        vi., 95% ethanol II for 1 h.
        vii., 100% ethanol I for 1 h.
        viii., 100% ethanol II for 1 h.
        ix., Histo-Clear I for 45 min.
        x., Histo-Clear II for 45 min.
        xi., Paraffin I for 1.5 h.
        xii., Paraffin II for 1.5 h.
    d. Select paraffin molds with an appropriate size for the tissues, dip the tissue in the molds filled with paraffin with the cut-side down, place them on the cold plate, wait for the paraffin to solidify, remove the paraffin blocks, and store them at 25°C (Figure 5C).

Pause point: Keep the harvested tissues in 4% paraformaldehyde for 48 h at 25°C. You can store the paraffin embedded tissues for extended periods.

    e. Trim the paraffin blocks using a rotary microtome until the entire vascular lumen structure is exposed, and immerse the blocks in an ice-water mixture for 2 h.
    f. Cut the paraffin tissues into consecutive 5 μm-thick slices and place them in 37°C warm water for spreading. Troubleshooting 3.

Note: Pay attention to the section with disrupted elastic plate of the aortic wall which should be included during slicing.

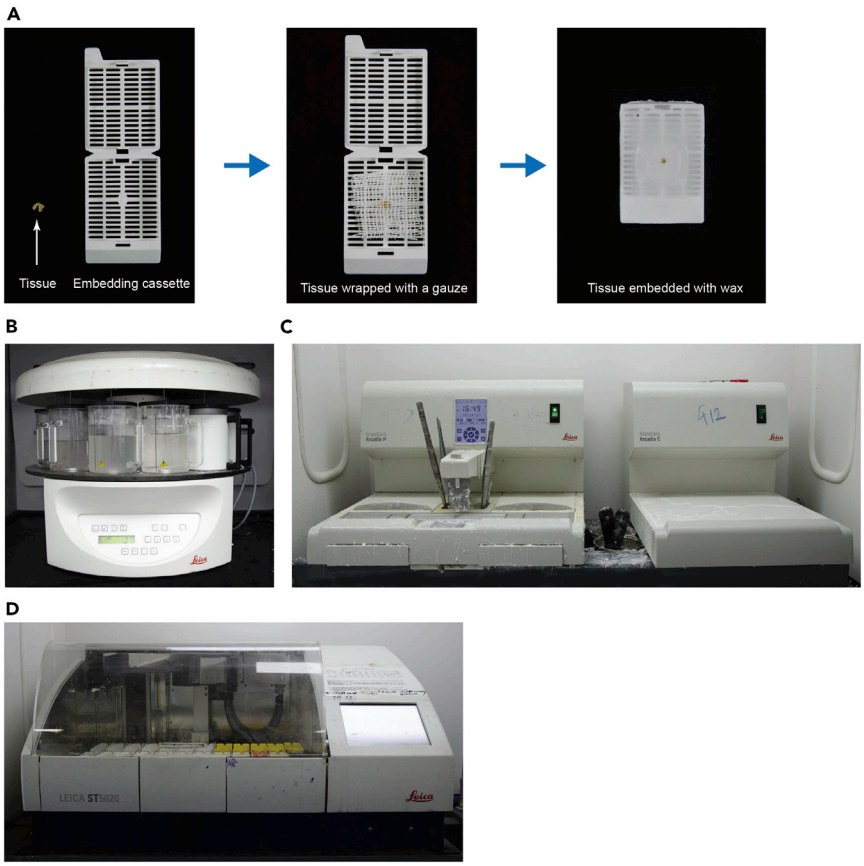

Figure 5. Instruments for paraffin section preparation and staining

(A) Tissue wrapped with a gauze in the embedding cassette.

(B) Automatic benchtop tissue processor.

(C) Heated paraffin embedding station and cold plate.

(D) Multifunctional slide stainer.

g. Gently pick up the tissue sections with adhesion microscope slides, put 2–3 pieces of tissue on each slide, drain residual water off the slide, place slides on the flattening table at 65°C for 2 h, put the slides into the section box and store them at 25°C.

Note: Label tissue groups on the slides with a pen that could not be removed in the aqueous or organic solvents, for example, a pencil.

19. Paraffin section dewaxing.

    a. Place sections in a multifunctional slide stainer (Figure 5D) and select the appropriate program for dewaxing as follows:

        i., 65°C for 30 min.

        ii., Histo-Clear I for 10 min.

        iii., Histo-Clear II for 10 min.

        iv., 100% ethanol for 5 min.

        v., 95% ethanol for 4 min.

        vi., 90% ethanol for 3 min.

        vii., 80% ethanol for 3 min.

        viii., 70% ethanol for 2 min.

        ix., water I for 5 min.

x., water II for 5 min.

b. Take out the dewaxed sections for the following staining.

20. Hematoxylin-Eosin staining.
    a. Place the dewaxed sections in hematoxylin staining solution for 3 min, and the nuclei are stained purple-blue under the microscope.
    b. Put the sections in water to wash out the remaining hematoxylin staining solution.
    c. Put the sections in alcohol containing 1% hydrochloric acid for differentiation, and the cytoplasm should be unstained while the nucleus should be stained blue-purple.
    d. Put the sections in running water for 0.5–1 min until the nucleus turned blue.
    e. Place the sections in eosin staining solution for 3–5 min until the cytoplasm is stained pink.
    f. Put the sections in water to wash out the remaining eosin staining solution.
    g. Put the sections in a multifunctional slide stainer for dehydration as follows:
        i., 80% ethanol for 5 s.
        ii., 95% ethanol for 1 min.
        iii., 100% ethanol I for 5 min.
        iv., 100% ethanol II for 5 min.
        v., Histo-Clear I for 5 min.
        vi., Histo-Clear II for 5 min.
    h. Seal the sections: add a drop of neutral balsam on one side of the section and slowly cover the coverslip to avoid air bubbles, or use Fully Automated Glass Coverslipper.
21. Elastic lamina staining.
    a. Place the dewaxed sections in working Elastic Stain Solution from Elastic (Connective Tissue Stain) Kit for 15 min.
    b. Put the sections in running tap water to wash out the remaining solution.
    c. Dip the sections in Differentiating solution from Elastic (Connective Tissue Stain) Kit for 15–20 times.
    d. Wash the sections in running tap water.
    e. Check the elastic fiber staining under the microscope, and repeat step c-d until the elastic fibers are clearly stained showing black-blue.
    f. Put the sections in Sodium Thiosulfate Solution for 1 min for de-iodizing.
    g. Wash the sections in running tap water.
    h. Stain the sections in Van Gieson's Solution from Elastic (Connective Tissue Stain) Kit for 2–5 min.
    i. Dehydrate the section as follows:
        i., 95% ethanol for 1 min.
        ii., 100% ethanol I for 5 min.
        iii., 100% ethanol II for 5 min.
        iv., Histo-Clear I for 5 min.
        v., Histo-Clear II for 5 min.
    j. Seal the sections: add a drop of neutral balsam on one side of the section and slowly cover the coverslip to avoid air bubbles, or use Fully Automated Glass Coverslipper.

EXPECTED OUTCOMES

EPO treatment for 4 weeks dose-dependently increases AAA incidence (20% for low-dose, 53.3% for medium-dose, and 60% for high-dose of EPO treatment) and abdominal aortic diameters in WT mice.[1] EPO injection for 4 weeks induces a dose-dependent increase in mortality in WT mice (6.7% for low-dose, 26.7% for medium-dose, and 40% for high-dose of EPO treatment).[1] Compared with normal saline treatment in the vehicle group, EPO injection increases aortic wall thickness and enhanced elastic lamina degradation in these mice (Figure 6).

LIMITATIONS

The high dose of EPO used in our animals is much larger than clinical doses of EPO for treating patients with anemia, although it is well known that animals exhibit a much higher tolerance to drugs

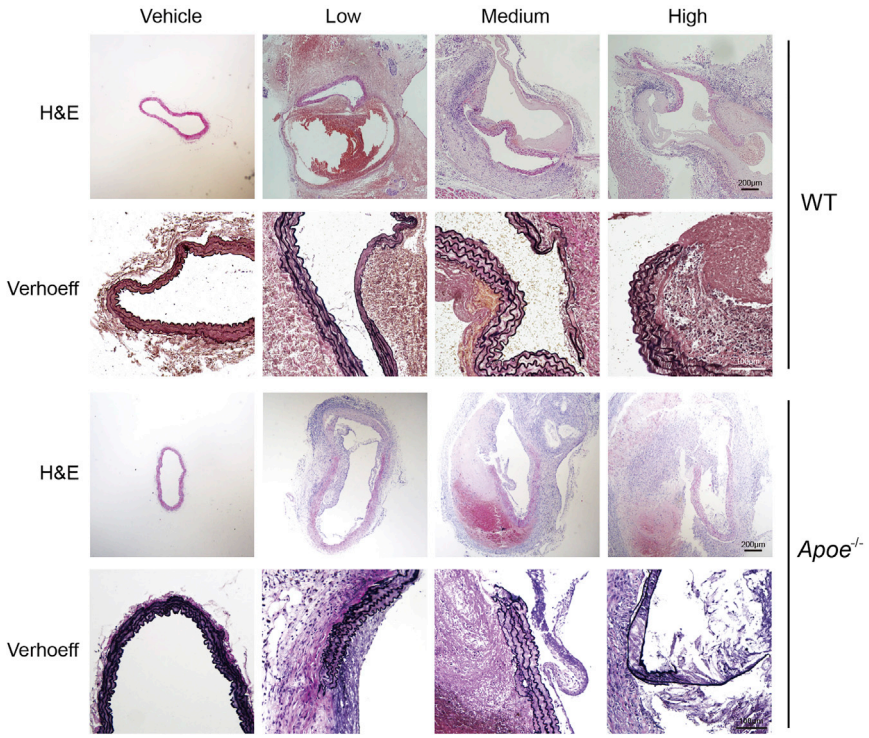

Figure 6. Representative H&E and Verhoeff staining of the abdominal aortic sections in WT and *Apoe*$^{-/-}$ mice receiving vehicle, low-, medium-, and high-dose of EPO treatment, respectively

H&E (scale bars, 200 μm) and Verhoeff (scale bars, 100 μm) magnifications are shown.

than human. However, the relative dose of EPO is much lower than that of AngII (the traditional AAA mouse model).

TROUBLESHOOTING

Problem 1

The incidence of AAA dose not reach the expected level (step 1).

Potential solution

If the AAA incidence is lower than expected, you should check whether EPO is protected from light in the process of preparation and injection. Totally dark is not necessary, but avoiding strong and straight light is essential.

Problem 2

Sufficient serum cannot be obtained from mice (step 10).

Potential solution

After EPO injection, the number of red blood cells per unit blood volume may significantly increase in mice, making it difficult to obtain sufficient serum if the collected blood is less than 1 mL. Use a 2 mL sterile empty needle to draw out about 1.5 mL of blood and then about 0.5 mL serum can be isolated for subsequent detection. A slight hemolysis is common.

Problem 3

Vacuolation or collapse of the vessel wall in sections of vascular tissue (step 18f).

Potential solution

When embedding, insufficient paraffin infiltration can lead to intravascular air bubbles or collapsed vessels, which will affect the subsequent observation and vessel wall measurement. Re-embedding of the vessel is recommended (step 18d).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact [Cheng Zhang] (zhangc@sdu.edu.cn).

Materials availability

All the materials used in this protocol are commercially available. The study did not generate new unique reagents and there are no restrictions to availability.

Data and code availability

All related information is available upon request from the lead contact. This study does not generate any code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101929.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (81920108003, 82030051, 81970373, 82200498, and 82000411), Key R & D Program of Shandong Province (2021ZLGX02, 2021SFGC0503, 2021ZDSYS05, 2020ZLYS05, and YXH2019ZXY004), the Shandong Natural Science Foundation (ZR2020YQ53 and ZR2020QH023), Program of Introducing Talents of Discipline to Universities (BP0719033), the Fundamental Research Funds of Shandong University (2018JC001), and Taishan Scholars Program of Shandong Province (M.Z., M.Z., and C.Z.).

AUTHOR CONTRIBUTIONS

Conceptualization, C.Z.; methodology, investigation, and writing – original draft, M.Z. (1st author), W.S.; writing – review & editing, Y.Z. and M.Z. (3rd author); funding acquisition, C.Z., Y.Z., and M.Z. (3rd author); supervision, Y.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Zhang, M., Sui, W., Cheng, C., Xue, F., Tian, Z., Cheng, J., Zhang, J., Zhang, T., Zhang, J., Wang, W., et al. (2021). Erythropoietin promotes abdominal aortic aneurysms in mice through angiogenesis and inflammatory infiltration. Sci. Transl. Med. *13*, eaaz4959. https://doi.org/10.1126/scitranslmed.aaz4959.

2. Zhang, X., Thatcher, S., Wu, C., Daugherty, A., and Cassis, L.A. (2015). Castration of male mice prevents the progression of established angiotensin II-induced abdominal aortic aneurysms. J. Vasc. Surg. *61*, 767–776. https://doi.org/10.1016/j.jvs.2013.11.004.

3. Thatcher, S.E., Zhang, X., Howatt, D.A., Yiannikouris, F., Gurley, S.B., Ennis, T., Curci, J.A., Daugherty, A., and Cassis, L.A. (2014). Angiotensin-converting enzyme 2 decreases formation and severity of angiotensin II-induced abdominal aortic aneurysms. Arterioscler. Thromb. Vasc. Biol. *34*, 2617–2623. https://doi.org/10.1161/ATVBAHA.114.304613.

4. Wang, L., Teng, R., Di, L., Rogers, H., Wu, H., Kopp, J.B., and Noguchi, C.T. (2013). PPARalpha and Sirt1 mediate erythropoietin action in increasing metabolic activity and browning of white adipocytes to protect against obesity and metabolic disorders. Diabetes *62*, 4122–4131. https://doi.org/10.2337/db13-0518.

5. Kent, K.C. (2014). Clinical practice. Abdominal aortic aneurysms. N. Engl. J. Med. *371*, 2101–2108. https://doi.org/10.1056/NEJMcp1401430.