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Research paper

Impact of adipose-derived stem cells on aortic tensile strength in a model of abdominal aortic aneurysm

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ABSTRACT

Introduction: Abdominal Aortic Aneurysm (AAA) is a highly morbid condition and is the 11th leading cause of death in the United States. Treatment options are limited to operative interventions, with minimal non-operative options. Prior literature has demonstrated a benefit to the use of mesenchymal stem cells (MSCs) in attenuating AAA formation. We demonstrate the utility of MSCs in treating AAA in swine, focusing on the mechanical and structural characteristics of aortic tissue after treatment.

Methods: 16 Yorkshire pigs underwent retroperitoneal exposure of the infrarenal aorta, with subsequent induction of AAA with peri-adventitial elastase and collagenase. A 1 × 4 cm piece of Gelfoam, an absorbable gelatin-based hemostatic agent, was soaked in media or human MSCs and placed directly on the vessel for control and experimental animals. At postoperative day 21, animals were sacrificed and the infrarenal aorta at this location was harvested for analysis. Tensile strength was measured using a tensiometer, from which Young's modulus and maximum strain were calculated.

Results: All animals survived the surgery and post-operative course. Young's elastic modulus for the aneurysm control group was 15.83 ± 1.61 compared to 22.13 ± 2.34 for the stem cell treated segment, $p = 0.0316$. There was no significant difference in the peak stress between groups.

Conclusions: This is the first study to demonstrate the mechanical effects of stem cell therapy on a model of AAA in swine. Young's modulus, which characterizes the intrinsic capacity of a tissue to withstand stress, was greater in the animals treated with MSCs compared to control animals with aneurysms. This methodology can be utilized in future large animal models to develop cell and drug-based therapies for AAA.

1. Introduction

Abdominal Aortic Aneurysm (AAA) is a highly lethal and morbid condition which is the 11th leading cause of death in the United States annually [1]. Consensus guidelines indicate the need for repair in asymptomatic fusiform abdominal aortic aneurysms >5.5 cm in diameter [2]. Treatment options for AAA include repair with open or endovascular surgery, which are each associated with their own complications and morbidity.

Aortic tissue is a complex, multi-layered structure with differing mechanical properties in each of its three layers: the intima, media, and adventitia. The intimal layer of the vessel is composed of a single layer of endothelial cells and, in a healthy aorta, is biomechanically insignificant [3]. In non-diseased vessels, the strength of the aorta is derived from the media and adventitia [4]. The medial layer is the thickest layer in normal aortic tissue, composed of smooth muscle cells, collagen, and elastin fibers in a complex arrangement. This layer is the main weight-bearing layer and is critical in generating the intrinsic compliance and resistance to flow changes [3,5]. The tunica adventitia is separated from the tunica media by a second elastic lamina. This adventitial layer is composed of extracellular matrix (ECM) proteins, particularly elastin and collagen fibers which generate the tensile strength required to contain the pressurized blood flowing from the heart, protecting the vessel from overstretching and rupture [5]. The pathophysiology of AAA formation in patients involves degeneration of the medial layer of the aortic wall, as well as an intense inflammatory response mediated by

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macrophage and neutrophil activation.

Prior literature has suggested a therapeutic benefit to the use of mesenchymal stem cells (MSC) in attenuating the inflammatory response via a reduction in the expression of CD4+ and CD8+ lymphocytes, and studies in small and large animal models of aortic aneurysm have demonstrated a therapeutic benefit to the administration of MSCs [6]. Work from our lab previously demonstrated an attenuation of aortic aneurysm formation in a model of aortic dilation in swine treated with human MSCs [10]. In this controlled study, we investigated the resulting alterations in mechanical properties of aortic tissue after stem cell treatment in a swine model of aortic disease.

2. Methods

2.1. Animal model and experimental design

Sixteen Yorkshire swine were utilized for this experiment and divided into two groups: eight animals in the aortic dilation only arm and eight animals in the stem cell + aortic dilation group, respectively. There were 14 castrated male pigs and 2 female pigs. All pigs were treated humanely according to guidelines set by the Cooper University IACUC. Aortic dilation was induced in all animals as previously described [7]. Briefly, A 5-cm left flank incision was made, followed by retroperitoneal exposure of the abdominal aorta. The left renal artery was identified as the anatomic distinction between the supra-renal and infra-renal aorta. Gross measurements with calipers were made at the infra-renal aorta as a control. A peri-adventitial injection of Type I collagenase (10,000 units/pig, Worthington Cat#: LS004196) and pancreatic porcine elastase (500 mg/pig, Sigma Chemical Co, Cat#: 400-146P) were made in the infra-renal segment of the aorta in all animals. In the aortic dilation control group, a 1 cm × 4 cm media-soaked piece of Gelfoam was placed on this section of treated aorta. In the stem cell + aortic dilation group, the Gelfoam was impregnated with media containing re-suspended 1×10^6 human adipose derived stem cells (H-ASCs). Gelfoam is a gelatin-based absorbable sponge material that is typically applied directly to tissue that is typically utilized as a hemostatic agent used to promote clot formation. In this experiment, we directly applied the foam to the infra-renal area of aorta that we measured, and the sample utilized was directly underneath this part of the aorta for our measurements. The incision was closed with 3-0 Vicryl and 4-0 Monocryl in layers, and the incision was closed with skin glue. The animals were kept in a closely monitored facility until postoperative day (POD) 21. On POD0, POD7 and 14, ultrasound was utilized to make measurements of the infra-renal aorta. The animals were sacrificed on POD21. During the sacrifice procedure, gross measurements of the aortic diameter were made, and aortic tissue was harvested for further tissue analysis and processing.

2.2. Stem cell isolation

Stem cells were isolated from solid tissue or lipoaspirate from patients at our institution undergoing elective plastic surgical procedures with their consent as per guidelines set by the Cooper University Hospital Institutional Review Board. Plastic surgery patients were selected as potential donors based on their age and general lack of comorbidities, as donor factors may play a role in stem cell efficacy [8]. Tissue was processed using protocols adapted from prior studies [9,10]. Briefly, either lipoaspirate or solid adipose tissue was obtained from clinical subjects. Solid tissue was mechanically minced into small pieces, rinsed with PBS, and filtered. These samples were then treated with Type 1 Collagenase and incubated at 37 °C for 1 h. Subsequently, the enzyme was inactivated with administration of M199 media. The stromal vascular fraction was then isolated in the aqueous fraction and centrifuged at 1000G for 5 min. The cell pellet was resuspended in media and purified through filtration through a 100 μm filter. The supernatant was discarded, and a small volume of sterile water was applied to aid in red blood cell lysis. M199 media was added, and the sample was filtered through a 40 μm filter. SVF was plated in T75 flasks in M199 media supplemented with 10 % fetal bovine serum. After 24 h, the media was aspirated, and adherent cells washed with PBS. Subsequently, media was changed every 2–3 days, and cells split and passaged per standard cell culture protocols. Flow cytometry was used to verify the pluripotent nature of these stems cells using markers CD44, CD90, CD73, CD105, and CD34. Cells were utilized in experiments between passage 3–5. For delivery of stem cells for the procedure, 1×10^6 were suspended in PBS for delivery into animal subjects.

2.3. Tensiometry measurements (Figs. 1 and 2)

We adapted previously described methodology to perform tensile strength measurements [7]. 1-cm ringed sections of infrarenal aorta were harvested and placed into sterile buffered saline prior to the measurements. Each ring of tissue was placed into a "s" hook and suspended by the tensiometer Shimadzu universal testing machine (EZ test, EZ-X, Shimadzu, Columbia, MD) (Fig. 1). A 500-N force sensor was used to conduct the mechanical test. The load range used was 0-30 N, and a displacement speed of 1 mm/min. Two aortic rings were measured for tensile strength per animal and averaged to account for any variance in diameter within the 4 cm segment. A stress-strain measurement curve was made for each tissue sample (Trapexium X material software).

2.4. Immunohistochemistry

Aortic specimens were collected from each animal and fixed in 10 % neutral buffered formalin for 48 h before being transferred to 70 % ethanol for storage. The tissue was transported to CVPath Labs (19 Firstfield Rd, Gaithersburg, MD 20878) to embed in in paraffin and generate 5 μm sections. Trichrome staining was performed utilizing the manufacturer's protocol (Abcam, ab150686). CVPath Labs performed additional staining with Verhoeff-van Gieson's stain (VVG) as a marker for elastin and for alpha-smooth muscle actin (α-SMA).

2.5. Statistical analysis

Paired *t*-tests were used to compare control and treatment measurements in a pairwise fashion, with the intention of limiting inter-subject variability. A Shapiro-Wilk test was used to confirm normality for continuous variables. Paired *t*-test was performed using GraphPad Prism and Microsoft Excel. A p-value <0.05 was deemed statistically

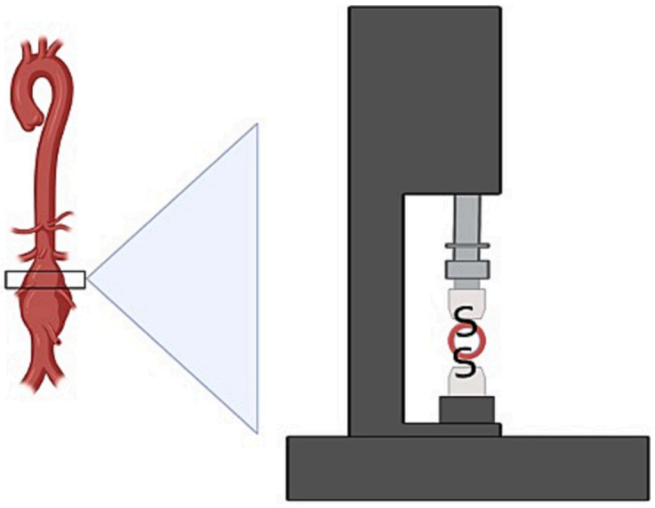

Fig. 1. Schematic of tensile strength measurements. One-centimeter rings of infra-renal aorta as demarcated by a box were sharply cut at time of sacrifice. Specimens were suspended on "S" hooks for tensile strength measurements.

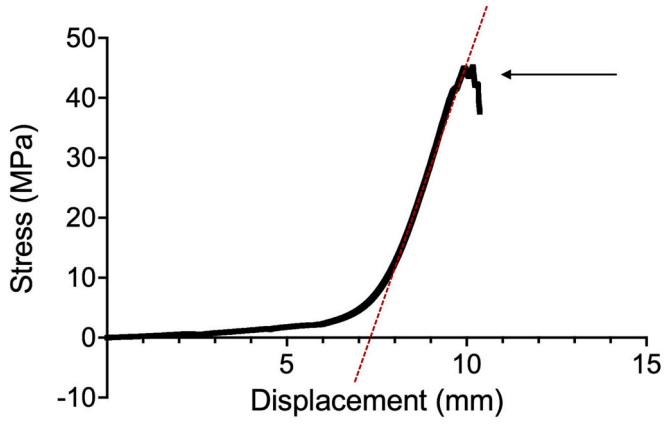

Fig. 2. Representative stress-strain curve utilized for tensile strength measurements. Dotted line denotes slope utilized for elastic modulus calculation. Arrow points to maximum tissue stress.

significant. Results are reported as mean ± standard deviation unless otherwise stated.

3. Results

Our protocol for inducing AAA in swine has been previously reported [7]. This method has proven to be reproducible and safe in subsequent experiments. All animals treated with stem cells survived to POD21. We previously demonstrated induction of aortic dilation in our control group, as well as an attenuation of this effect via administration of H-ASCs [10].

3.1. Tensile strength calculations

The uniaxial stress test was completed when the force applied failed to translate into a change in displacement. A stress-strain curve was generated for each tissue specimen (Fig. 2). Maximum stress was calculated as the peak force at the apex of the curve. Young's modulus was calculated as the slope of the stress-strain curve (Newtons/mm^2).

3.2. Tensile strength measurements (Figs. 3 and 4)

The elastic modulus was calculated based on the slope of the curve generated for each specimen using a short segment of the curve which is nearly linear (Fig. 3). The elastic modulus for the aortic dilation control group was 15.83 ± 6.434 compared to 22.13 ± 8.762 for the stem cell treated segment ($p = 0.0316$). Peak stress in the infra-renal aorta from the explanted tissue was 16.78 ± 5.629 in the aortic dilation control group versus 17.93 ± 5.357 in the aortic dilation + stem cell treated animals ($p = 0.5643$) (Fig. 4).

3.3. Histological analysis of aortic tissue (Fig. 5)

We used Masson's Trichrome, Verhoeff-van Gieson, and α-SMA staining to characterize the structure of collagen, elastin and smooth muscle for aortic dilation control animals compared to the aortic dilation + stem cell treatment group (Fig. 5). In aortic dilation group, there was a degradation and decreased quantity of these structural fibrils [10]. In addition to the degeneration of collagen, elastin and smooth muscle, the aortic dilation group was noticeably perturbed compared to the specimens treated with stem cells.

4. Discussion

This study is the first of its kind to demonstrate the mechanical effects of the treatment of abdominal aortic aneurysm with human ASCs.

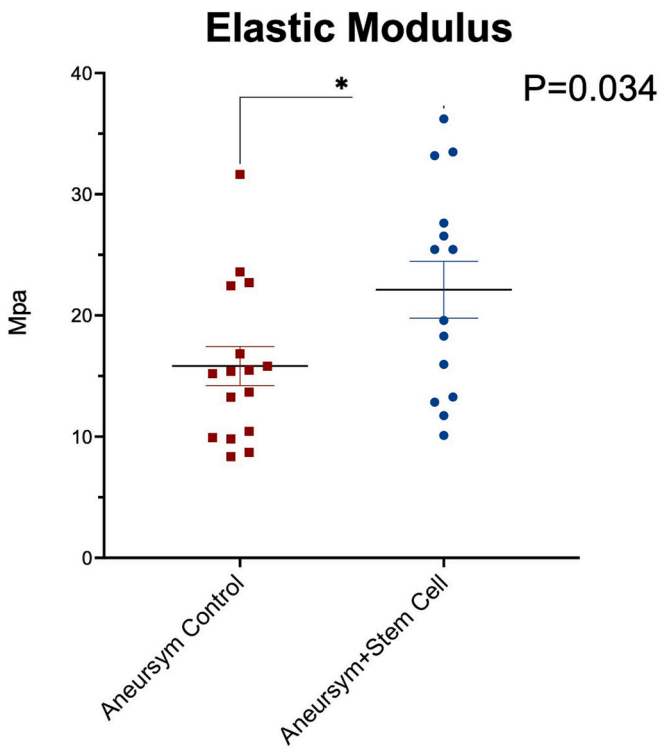

Fig. 3. Elastic modulus measurement comparison between infra-renal aorta samples from untreated dilated aorta (15.83 ± 6.434) and stem-cell treated aortic tissue (22.13 ± 8.762) at 21 days after enzymatic digestion. ($p = 0.0316$).

Prior studies have demonstrated that the biomechanical properties of AAA are deranged both during the phase or aneurysmal dilation, as well as in rupture [11]. Mechanically, the inflammatory process underlying AAA formation is wall weakening, associated with dilation of the aorta until rupture, which occurs when the stress on the tissue exceeds the strength of the vessel wall. This property is described classically by the law of Laplace, which describes the wall tension of the vessel to be proportional to the transluminal pressure and radius of the vessel, and inversely proportional to the thickness of the wall. Prior studies have demonstrated that the aortic wall of diseased tissue is less distensible and stiffer than non-diseased tissue, mediated by derangement in the organization of the underlying collagen and elastin structure in the tissue [12]. Young's elastic modulus, as well as maximum strain measurements, have been utilized previously to characterize the underlying structural characteristics of aortic aneurysms [13]. We utilized these same techniques to characterize the impact of the treatment of MSCs on a porcine model of induced aortic dilation. Our results show a reduction in the Young's modulus in the diseased infrarenal aorta that correlates to prior studies of aortic aneurysm in porcine tissue [14,15]. However, with stem cell therapy, this change was ameliorated which does correspond to the preservation of the structural proteins with cell therapy that we noted [10].

Our technique of inducing aortic dilation in a large animal model has been reproducible and technically feasible [7,10]. In those investigations, ultrasound and histologic evaluation were utilized to demonstrate induction of aortic dilation as well as a clear prevention of aortic dilation associated with treatment using stem cells. In this investigation, we build on these investigations to provide a deeper mechanistic understanding of the manner in which stem cell therapy works to minimize the effects of aortic aneurysm. De Leo et al. demonstrated in a model of aortic aneurysm induced by enzymatic degradation that there was decrease in the Young's modulus in the aneurysmal tissue that was not seen in the untreated adjacent aortic

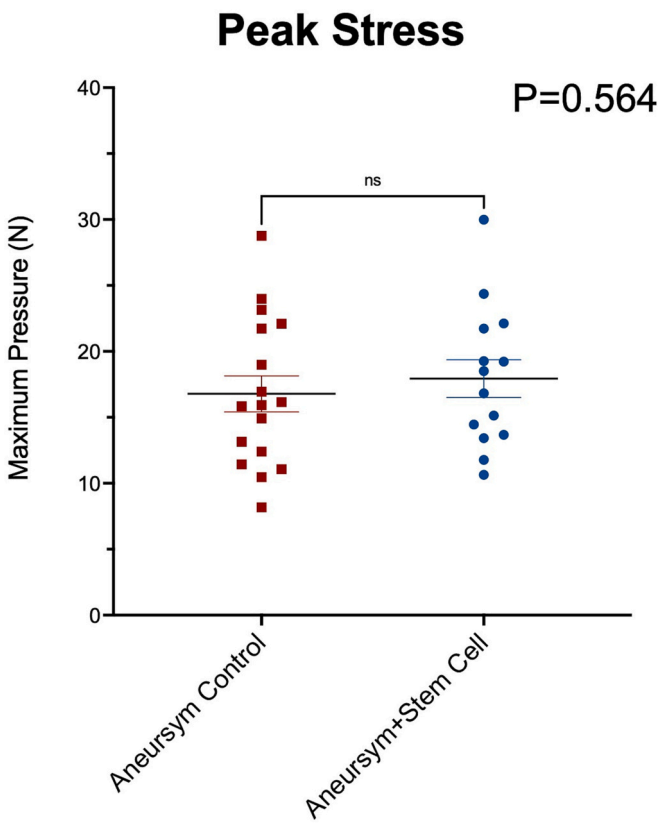

Fig. 4. Comparison of peak stress measurements (the peak of the stress-strain curve) between infra-renal aorta samples from untreated dilated aorta (16.78 ± 5.629) and stem-cell treated aortic tissue (17.93 ± 5.357) at 21 days after enzymatic digestion. ($p = 0.5643$).

control tissue [7]. In this study, we again demonstrated there was a reduction in the elastic modulus of the aortic dilation control group; but importantly, we also found that the biomechanical properties were preserved in the stem cell treated animals despite enzymatic degradation.

Histologically, AAA is associated with fragmentation of elastin fibrils and perturbation of collagen within the aortic media and ECM. Prior studies have demonstrated a decrease in the overall content of collagen in aneurysmal tissue, but with increased amounts of cross-linking suggesting that the tissue simultaneously becomes thinner and stiffer, reducing the distensibility of the vessel wall [16]. We did not see a difference in overall peak stress of the vessel wall with stem cell administration, which may be reflective of these factors. Recent studies have started to investigate the micromechanical biomechanical properties of healthy and diseased aortic tissues. Future investigations to provide an individual assessment of the biomechanical properties of each layer of the vessel wall may be valuable [17].

There is a regional difference in the composition of these molecules within the vessel wall throughout its anatomic course. The composition and orientation of these structural components changes spatially distally from the origin of the aorta down to the infra-renal segment, which likely is a reflection on the pressure differential that the tissue sees. There is a increase in the quantity of collagen protein in the distal aorta compared to the proximal aorta [18]. Additionally, there is a large amount of elastin fibers in the proximal ascending aorta, which is likely an adaptive mechanism to the high pressures seen from left ventricular outflow. Patients with connective tissue disorders of collagen such as Ehlers Danlos syndrome and Marfan's disease are also predisposed to aortic dissections and aortic aneurysm formation, which also indicate the importance of these structural components on AAA formation. Multiple studies have described a loss of collagen type I and collagen type III in the late stages of aneurysmal disease [19]. In human aortic tissue, there is re-organization of the density and composition of these structural proteins in aneurysm disease. In the adventitia of healthy aortic tissue, collagen is organized as a loose knit structure, which allows it the ability to distend in response to changes in pressure. However, in aneurysmal disease, collagen fibrils run in a parallel orientation, minimizing the distensibility of the tissue [20]. The elastic modulus in tissue is generally considered based on the organization of the structural fibrils of these proteins, and the modulation of the individual structure protein components by stem cell therapy may ultimately be the driving force behind the functional changes that we observed.

Our study does have its limitations. The improved tensile strength of the aorta after stem cell treatment suggests that stem cells in the peri-adventitia space have a local effect, either from direct engraftment or through activation of signaling pathways to minimize the deleterious effects of the treatment cocktail. We did not identify the presence of the stem cells at POD21, but prior investigations suggest that the beneficial effects of stem cell treatment occur prior to this time frame [21,22]. In our experiment, we utilized gelfoam as a vector to place the stem cells suspended on media directly on the aortic tissue. While we considered additional cell delivery methods including direct injection into the vessel wall, we did not want to induce any iatrogenic injury to the tissue. We found that using this gelatin, porcine based hemostatic agent was easily performed and did not lead to any vessel injury. Notably, we used a combination of castrated male and female pigs, and the differential in aortic size between different gender of swine is relatively new and would be important to consider in further studies. Future studies must be performed to investigate the signaling pathways and changes in gene regulation that result in the protective effects on aortic tissue that we identified. Additionally, we decided to use human derived mesenchymal stem cells in our experiments, rather than porcine derived cells. Prior studies have demonstrated the regenerative and reparative abilities of this specific mesenchymal cell population [23,24]. Additionally, this cell type has been used in various other scaffold structures involved in cardiovascular remodeling [25,26]. From a practical standpoint, we found that the isolation of porcine adipose tissue from adolescent animals is technically difficult as they contain very little flank adipose tissue.

We only utilized circumferential sections of aortic tissue for our tensile strength measurements. Aortic tissue exhibits anisotropic mechanical characteristics, and an accurate quantification of the mechanical properties would ideally be measured in the longitudinal and circumferential direction [13]. In our model, we utilized only circumferential measurements of tensile strength, as we were limited by the quantity of aortic tissue available due to the relatively short infra-renal aortic length of the vessel. As a limitation, the force measured by uniaxial testing does not estimate the radial force felt by the aorta, which is likely the primary physiologic direction of force the vessel resists to prevent rupture. Therefore, this methodology does not provide an overall measure of tensile strength, but we believe that it is a useful adjunct to other testing methodologies that can help characterize the impact of therapeutic agents on aneurysm formation. While we displayed a significant change in the mechanical properties of stem-cell treated aorta compared to diseased aorta, we did not have enough normal aortic tissue for a direct comparison to stem-cell treated tissue. We intend to include normal untreated aortic tissue in future studies. However, the overall objective of our study was not to demonstrate an absolute characterization of the mechanical property of the porcine aorta, but rather to utilize these properties to better understand the role of stem cell therapy. Additionally, we envision that this methodology can be utilized in future investigations as a sensitive way to test future drug and/or cell-based therapies for AAA.

This is the first study to demonstrate the mechanical effects of stem cell therapy on a model of AAA in swine. Young's modulus, which characterizes the functional capacity of a tissue to withstand stress, was preserved in the animals treated with MSCs compared to control animals with aneurysms. This methodology can be utilized in future large animal

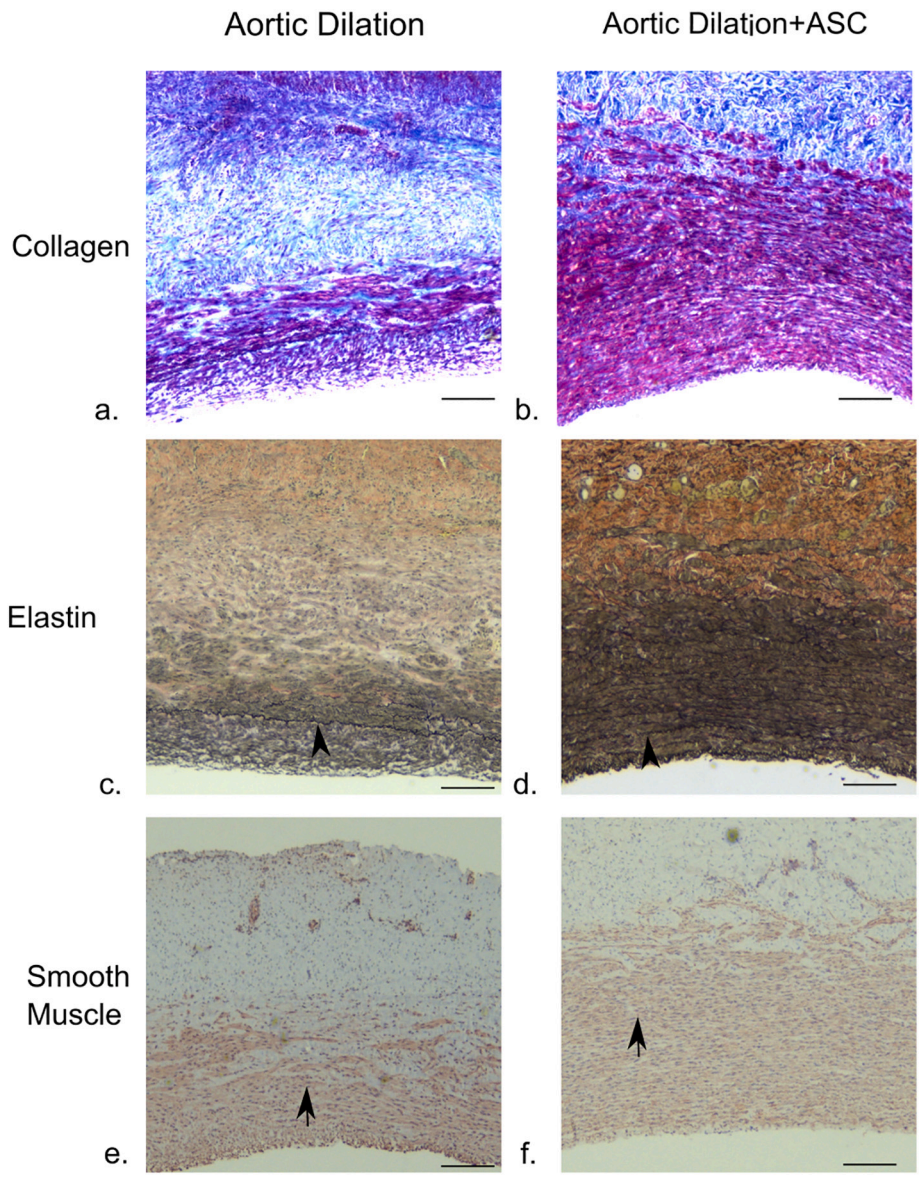

Fig. 5. Histological analysis of aortic specimens from untreated animals with dilated aortas (a, c, e) compared to those treated with stem cells (b, d, f) showed preservation of collagen (blue staining), elastin fibrils (arrowhead), and smooth muscle (arrow) in the treatment group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

models to develop additional cell and drug-based therapies for AAA.

CRediT authorship contribution statement

Keshav Kooragayala: Investigation, Writing – original draft, Visualization, Formal analysis, Writing – review & editing. **Johanna Lou:** Investigation, Writing – original draft, Visualization, Formal analysis, Writing – review & editing. **Vaishali Krishnadoss:** Investigation, Software, Formal analysis, Writing – review & editing. **Brian Zilberman:** Investigation, Formal analysis. **Nicholas Deleo:** Investigation, Formal analysis. **Olga Ostrovsky:** Investigation, Resources, Writing – review & editing. **Ping Zhang:** Investigation, Resources, Writing – review & editing. **Iman Noshadi:** Conceptualization, Methodology, Resources. **Spencer Brown:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Jeffrey P. Carpenter:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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