Clinical Research in Cardiology (2019) 108:847–856
https://doi.org/10.1007/s00392-019-01413-1

ORIGINAL PAPER

Graded murine wire-induced aortic valve stenosis model mimics human functional and morphological disease phenotype

Sven Thomas Niepmann[1] · Eva Steffen[1] · Andreas Zietzer[1] · Matti Adam[2] · Julia Nordsiek[1] · Isabella Gyamfi-Poku[3] · Kerstin Piayda[3] · Jan-Malte Sinning[1] · Stephan Baldus[2] · Malte Kelm[3,4] · Georg Nickenig[1] · Sebastian Zimmer[1] · Christine Quast[3]

Received: 5 November 2018 / Accepted: 14 January 2019 / Published online: 14 February 2019

Abstract

Aortic valve stenosis (AS) is the most common valve disease requiring therapeutic intervention. Even though the incidence of AS has been continuously rising and AS is associated with significant morbidity and mortality, to date, no medical treatments have been identified that can modify disease progression. This unmet medical need is likely attributed to an incomplete understanding of the molecular mechanism driving disease development. To investigate the pathophysiology leading to AS, reliable and reproducible animal models that mimic human pathophysiology are needed. We have tested and expanded the protocols of a wire-injury induced AS mouse model. For this model, coronary wires were used to apply shear stress to the aortic valve cusps with increasing intensity. These protocols allowed distinction of mild, moderate and severe wire-injury. Upon moderate or severe injury, AS developed with a significant increase in aortic valve peak blood flow velocity. While moderate injury promoted solitary AS, severe-injury induced mixed aortic valve disease with concomitant mild to moderate aortic regurgitation. The changes in aortic valve function were reflected by dilation and hypertrophy of the left ventricle, as well as a decreased left ventricular ejection fraction. Histological analysis revealed the classic hallmarks of human disease with aortic valve thickening, increased macrophage infiltration, fibrosis and calcification. This new mouse model of AS promotes functional and morphological changes similar to moderate and severe human AS. It can be used to investigate the pathomechanisms contributing to AS development and to test novel therapeutic strategies.

Keywords Aortic valve stenosis · Animal models · Inflammation · Calcification

Introduction

Aortic valve stenosis (AS) is one of the most common valve diseases in the western world [1]. The natural history of AS is slow and progressive, and is characterized through a long asymptomatic period. Once patients develop symptoms such as dyspnea, angina or syncope, AS becomes life threatening and mortality increases to 50% within 2 years [2, 3]. Common risk factors such as hypertension, dyslipidemia, male sex, smoking and diabetes have been linked to AS, but they are not highly predictive [4, 5]. The first sign of AS development is increased sclerosis of the aortic valve, but only 10–15% of these patients develop AS with a critical reduction in valve orifice area. Once a mild or moderate stenosis is detected, nearly 100% of patients eventually develop severe aortic valve stenosis [6, 7]. Currently, there are no medical interventions efficacious in altering disease progression

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00392-019-01413-1) contains supplementary material, which is available to authorized users.

✉ Sven Thomas Niepmann
sven_thomas.niepmann@ukbonn.de

[1] Heart Center Bonn, Clinic for Internal Medicine II, University Hospital Bonn, Bonn, Germany

[2] Clinic for Cardiology, University Hospital Cologne, Cologne, Germany

[3] Cardiovascular Research Laboratory, Division of Cardiology, Pulmonary Diseases and Vascular Medicine, Medical Faculty, Heinrich-Heine University, Düsseldorf, Germany

[4] CARID, Cardiovascular Research Institute Düsseldorf, Düsseldorf, Germany

or reliable markers that predict disease development, the future need for surgery or mortality. Patients with AS are merely monitored for disease progression and, in the presence of symptoms or left ventricular dysfunction, undergo aortic valve replacement [8]. Over the past decade, the number of aortic valve replacements has doubled and is likely to double again within the next 20 years [1], due to an ever increasing aging population.

Because of similar clinical risk factors, the pathophysiology of AS has often been associated with atherosclerosis. Nevertheless, major differences in clinical presentation and therapeutic outcome underline the distinct characteristics of atherosclerosis and AS. First, the incidence of patients with AS who also have significant coronary artery disease is only approximately 50–60% [9–13]. Second, treatment with HMG-CoA-reductase-inhibitors (statins) is ineffective in preventing AS progression, whereas the benefits of statins in patients with atherosclerosis are well documented [14, 15]. Third, prominent single-nucleotide polymorphisms associated with AS are independent of coronary artery calcification and clinical coronary artery disease [16].

AS has traditionally been considered a passive consequence of degenerative processes, caused by decades of mechanical stress [17]. However, evidence from explanted human aortic valves has rebutted this outdated notion and our current understanding of AS holds overactive chronic inflammation responsible for disease development [18]. At the core of this process are cellular and biochemical pathways, which are orchestrated through the activation of immune mechanisms, changes in interstitial cell function and matrix remodeling [17, 19]. However, most of these observations only reflect advanced disease stages, and disease progression and early development remain elusive. This eminent gap between significant medical need and poor understanding of AS pathophysiology is, to a relevant amount, explained by the lack of appropriate AS animal models.

In 2014, a new model for aortic valve stenosis was published by Honda et al. Here, the aortic valve endothelium was mechanically damaged using a spring wire. The wire was inserted into the left ventricle and moved in and out 20 times. Then, the wire was positioned on valve level and rotated 50 times. This resulted in the development of AS within 1 week after surgery [20].

While trying to establish this wire-induced AS mouse model in our laboratory, we had difficulty to induce consistent and reproducible AS when adhering precisely to the given instructions. We have, therefore, modified and expanded upon the original protocols, and have developed and characterized distinct models with mild, moderate and severe wire-injury.

Methods

Mice

10–12 weeks old male C57BL/6-J (wild-type) mice were purchased from Janvier Labs, France. Animals were maintained in a 22 °C room with a 12-h-light/dark cycle and received chow and drinking water ad libitum. All animal experiments were performed according to institutional guidelines and the German animal protection law.

Echocardiography

For all functional analysis, a Fujifilm Visualsonics Vevo 2100 Ultra High Frequency Imaging Platform was used. Mice were anesthetized with 1.5% isoflurane with continuous monitoring of electrocardiogram, respiratory rate and body temperature. The chest of all mice was depilated with chemical hair remover to improve image quality.

Aortic valve peak velocity was measured in the suprasternal view with a pulse-wave-Doppler using angle correction between 40° and 50°. An increase in peak velocity of 15–50% from baseline was defined as a mild, 50–75% as a moderate and > 75% as a severe aortic valve stenosis.

Left ventricular ejection fraction, fractional shortening and ventricular volumes were measured in parasternal long-axis views using the Vevo LV-Trace function. Wall thicknesses were measured in parasternal long- and short-axis M-Modes. Aortic valve regurgitation was imaged using color-Doppler mode in the parasternal long-axis and suprasternal views.

Aortic valve injury

The mice were anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 16 mg/kg xylazine. The right carotid artery was exposed by blunt dissection and blood flow was stopped using ligature loops. For mild and moderate injury, a straight guide wire with a shortened and soldered tip (Abbott HI-TORQUE 0.014″) was used. For severe injury, a conventional guide wire with a 15° angled tip (ASAHI INTECC MIRACLEbros 6) was used. The wire was introduced into the left ventricle under echocardiographic guidance, passed over the aortic valve and advanced into the left ventricular apex and pulled back into the left ventricular outlet, just below the aortic valve level. This resulted in an amplitude of 4–5 mm. Hereafter, the wire was rotated over the valve with a speed of two rotations per second as described below.

After injury, acute aortic valve regurgitation was immediately assessed using a color-Doppler. The wire was

removed and the carotid artery was ligated. Sham procedure was performed in the same fashion, but the wire was only inserted into the right carotid artery and not advanced across the aortic valve into the left ventricle.

Histological analysis

The mice were euthanized via cervical dislocation 4–8 weeks after moderate or severe aortic valve injury. Hearts were flushed with 0.9% sodium chloride solution, embedded in tissue freezing medium and sectioned (8 µm thickness). Sections were stained with hematoxylin and eosin according to standard protocols. Tissue calcification was measured with von Kossa staining (Abcam Staining Kit). Collagen was visualized using Pico-Sirius-Red Staining (Sigma-Aldrich). Images were obtained using light/polarization (Sirius-Red) microscopy at 10x (Axio Observer, Zeiss, Germany). Aortic valve area, collagen and calcium deposits were measured with Zeiss ZEN Imaging Software.

Immunofluorescence

Macrophage infiltration into stenotic aortic valves was measured via CD68 staining. Sections were fixed in acetone for 20 min, washed with phosphate buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) for 30 min. The slides were then incubated with the primary antibody at 4 °C over night. The primary antibody was diluted 1:100 (anti-CD68 rat IgG2a, Acris Antibodies, Germany). The sections were washed again using PBS and incubated with the secondary antibody, diluted 1:50 (Cy3 AffiniPure Donkey anti Rat IgG, Jackson ImmunoResearch Laboratories Inc) for 1 h at 4 °C. After washing with PBS, nuclei were counterstained with Vecatshild mounting medium containing 4′,6′-diamidino-2-phenylinodole (DAPI) (Vector Laboratories).

Apoptosis was visualized using an "in situ cell death detection kit-Fluorescin" (Sigma-Aldrich). Briefly, slides were blocked in 4% paraformaldehyde (PFA) in PBS pH 7.4 for 1 h and incubated in permeabilization solution containing 0.1% Triton X and 0.1% sodium citrate. Afterwards, TdT-mediated dUTP-biotin nick end labeling (TUNEL) reaction mixture was added for 1 h. Positive controls were treated with DNase I for 15 min prior to incubation with the TUNEL-reagent. Slides were then washed with PBS and nuclei were counterstained using Vectashield mounting medium containing DAPI.

Images were taken with an Axio Observer (Zeiss, Germany). The CD68-positive areas were automatically quantified with Zeiss ZEN Imaging Software.

Human specimens

Aortic valve specimens were collected from patients undergoing surgical aortic valve replacement for either severe aortic stenosis or aortic regurgitation. All patients provided written informed consent. The study protocols were approved by the local ethics committee.

After fixation in formaldehyde for 24 h the valves were decalcified using Titriplex III-buffer (Merck) for 72 h, paraffin embedded and sectioned (4 µm thickness). Sections were stained with hematoxylin and eosin according to standard protocols.

Macrophage infiltration was measured via anti-CD68 staining. Sections were rehydrated, boiled in citrate buffer for 20 min, washed with PBS and blocked with 0.5% BSA for 30 min. The slides were then incubated with the primary antibody at 4 °C over night. The primary antibody was diluted 1:1000 (anti-CD68 rabbit IgG, Abcam, USA). The sections were washed again using PBS and incubated with the secondary antibody, diluted 1:50 (Cy2-Goat anti Rabbit IgG, Abcam, USA) for 1 h at 4 °C. After washing with PBS, nuclei were counterstained with Vectashild mounting medium containing DAPI (Vector Laboratories). Images were taken with an Axio Observer (Zeiss, Germany).

Statistical analyses

All data are presented as mean ± SEM. Statistical significance was calculated by one-way ANOVA followed by Tukey test for multiple comparisons. Two groups were compared using Student's *t* test. *P* values of 0.05 or less were considered to be statistically significant.

Results

Variation in wire type, tip angle and number of rotations leads to distinct AS intensities

When adhering precisely to the protocols published by Honda et al., we were unable to induce significant AS in mice. The first challenge in the induction of consistent and reliable results is the precise wire placement and movement within the very small mouse anatomy and high heart rate. To increase the stability and exact positioning of the wire, we designed and fabricated a stand consisting of a torquer and a catheter insertion tool. This stand greatly reduced uncontrolled lateral movement and prevented dislocation out of the left ventricle while rotating on the valve level. Next, the number of rotations on valve level was increased progressively to 200 to attain the increase in aortic valve peak velocity described by Honda et al. With these improvements, we were able to induce a severe aortic valve stenosis within

1 week after surgery. Unfortunately, this also resulted in a mortality rate of 15% within the first 24 h after surgery, due to severe aortic valve regurgitation. While this model is sufficient for studying advanced mixed aortic valve disease, a more moderate injury model and a pure aortic valve stenosis are required to investigate the development of aortic valve stenosis. Thus, to improve survival and promoted pure aortic valve stenosis we adapted the protocols further. Injury intensity was reduced by modulating the wire type, tip angle and the number of rotations. Through gradually adaption of the protocol, we have now developed three distinct models of wire-induced aortic valve injury. For "mild" and "moderate" aortic valve stenosis induction, a guide wire with a straight tip was used. The wire was passed over the aortic valve and pushed back and forth 20 times in the mild and 50 times in the moderate model. The wire was then rotated 50 times in the center (mild injury) or 100 times in direction of all aortic valve cusps (moderate injury) (Fig. 1a, c). For the "severe" stenosis model, a coronary wire with a 15° angled tip was used, pushed back and forth 20 times over the aortic valve and then rotated 200 times (Fig. 1b, d).

One week after surgery, a significant increase in aortic peak velocity was consistently measured upon moderate and severe injury (Fig. 1e). The mild injury model only induced a mild increase in peak velocity compared to baseline or sham-operated mice (Fig. 1e). In contrast to the severe injury, the mortality within the first 24 h upon mild and moderate injury was down to 4.4%. Similarly, the incidence of acute aortic regurgitation was also lower. Upon mild injury, aortic regurgitation could not be detected, while moderate injury led to 4% and severe injury led to 7% incidence of post-surgical aortic valve regurgitation (Fig. 1f).

Graded injury results in graded aortic valve stenosis

To evaluate the longitudinal change in peak velocity, weekly two-dimensional color-Doppler and pulse-wave-Doppler imaging was performed. After 1 week, peak velocity (Fig. 2a), peak gradient (Fig. 2b) and mean gradient (Fig. 2b) were significantly increased in both moderate and severe injury models compared to baseline and sham-operated control mice. At 1 week, there was no difference between moderate or severe injury models. After 4 weeks, however, the peak velocity upon moderate injury remained stable while it continued to increase in severe-injured mice (Fig. 2a, d). In the same manner, changes in peak and mean trans-aortic gradients were registered (Fig. 2b, d). Peak velocity of aortic valve blood flow plateaus after 4 weeks and no further increase can be detected in longer observations (Supplemental Fig. 1A). Even after 12 weeks, the

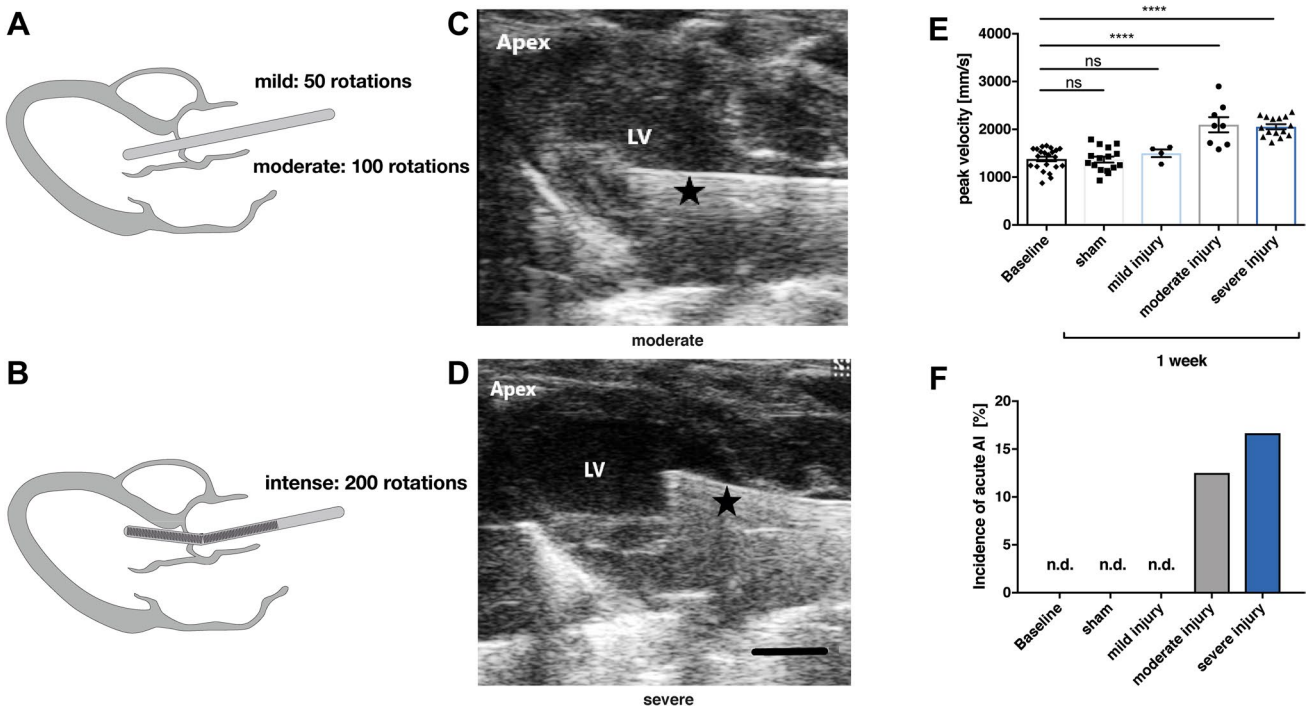

Fig. 1 Modified protocol of graded wire-injury induced aortic valve stenosis in mice: For mild and moderate injury a straight guide wire is pushed in and out over the valve 20 or 50 times, and rotated 50 or 100 times, respectively (**a**). For severe injury, a spring wire with an angled tip of 15° (**b**) is pushed in and out over the aortic valve a total of 20 times and rotated 200 times. Exemplary parasternal long-axis views of straight (**c**) or angled wire (**d**). Aortic valve peak velocities 1 week after mild, mild, moderate or severe injury compared to sham procedure (**e**). Incidence of acute post-surgical aortic valve regurgitation (**f**)

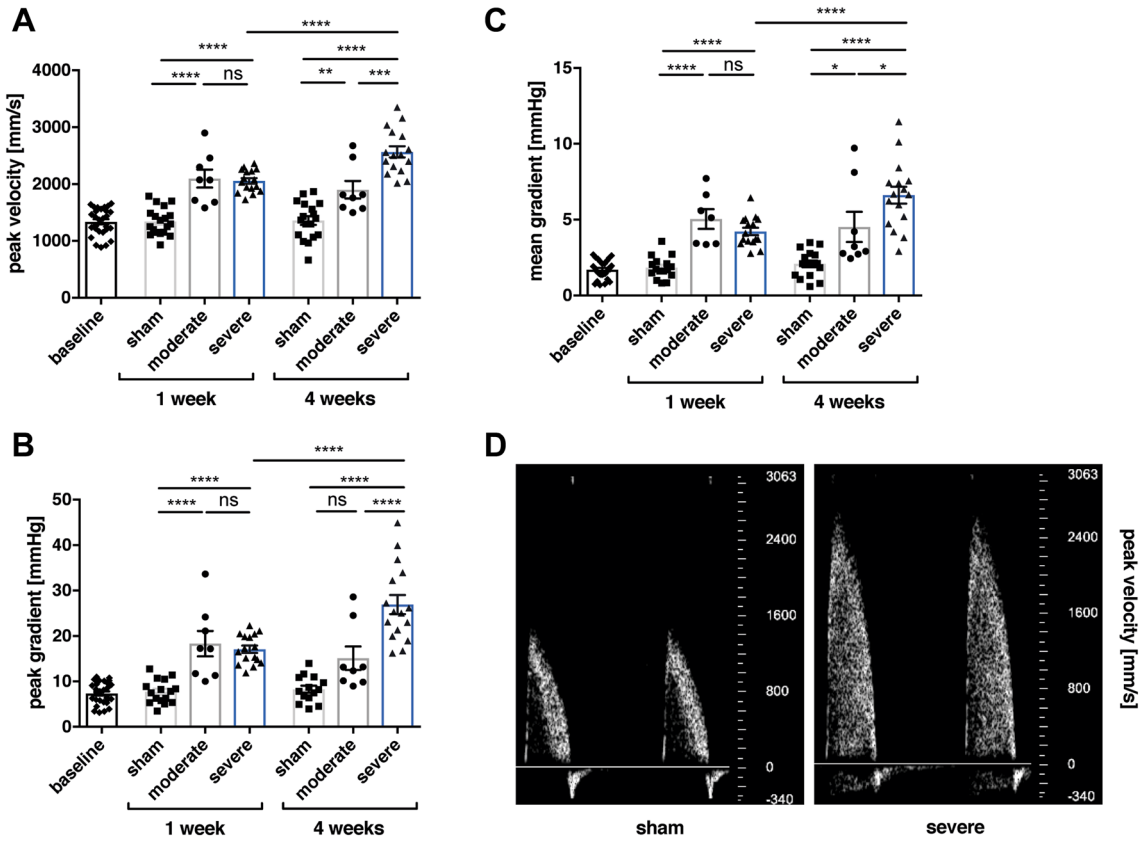

Fig. 2 Aortic blood flow after wire-injury: Aortic valve blood flow peak velocity (**a**), peak gradient (**b**) and mean gradient (**c**) of moderate ($n = 11$) and severe-injured ($n = 16$) mice compared to sham-mice ($n = 16$). Representative Doppler profiles of sham-mice and mice with severe injury 4 weeks after surgery (**d**)

trans-aortic peak velocity remained stable in moderate or severe-injured mice compared to 4 weeks after wire-injury (Supplemental Fig. 1B).

Mice subjected to severe injury develop aortic regurgitation

Patients with severe AS often suffer from mixed aortic valve disease. In fact, in our collective of patients undergoing transcatheter-aortic-valve-replacement (TAVR) mild, moderate and severe aortic regurgitation are observed in 55%, 17% and 2.2%, respectively [21]. We, therefore, studied the incidence of aortic regurgitation 4 weeks after wire-induced aortic valve injury using color-Doppler in parasternal long-axis views. We defined a small jet restricted to close proximity of the aortic valve as mild, a larger jet stretching the length of the left ventricular outlet tract as moderate and a jet reaching into the left ventricle as severe aortic regurgitation (Fig. 3a). Only 11.25% of the mice that received a moderate injury displayed an aortic regurgitation. In the group of mice with severe injury, 50% of the mice had a mild and 18.75% had a moderate aortic insufficiency. Severe aortic regurgitation was not detected in any of the examined mice (Fig. 3b).

To assess if the observed aortic regurgitation had impact on left ventricular (LV) dimension, we measured LV volume in the parasternal long-axis view. Indeed, the systolic and diastolic LV volume was significantly increased in severe-injured mice 4 weeks after surgery. LV volumes of mice with moderate injury, which only showed a low rate of aortic regurgitation, remained unchanged (Fig. 3c, d).

Mixed aortic valve disease upon severe injury leads to impaired left ventricular function

LV ejection fraction and longitudinal fractional shortening were measured in the parasternal long-axis view. No differences in ejection fraction could be seen 1 week after either moderate or severe injury when compared to sham-mice. After 4 weeks, a significant drop in LV ejection fraction and fractional shortening in severe-injured mice compared to moderate-injured and to sham-mice was measured (Fig. 4a). Fractional shortening in severe-injured mice already decreased 1 week after injury and remained stable

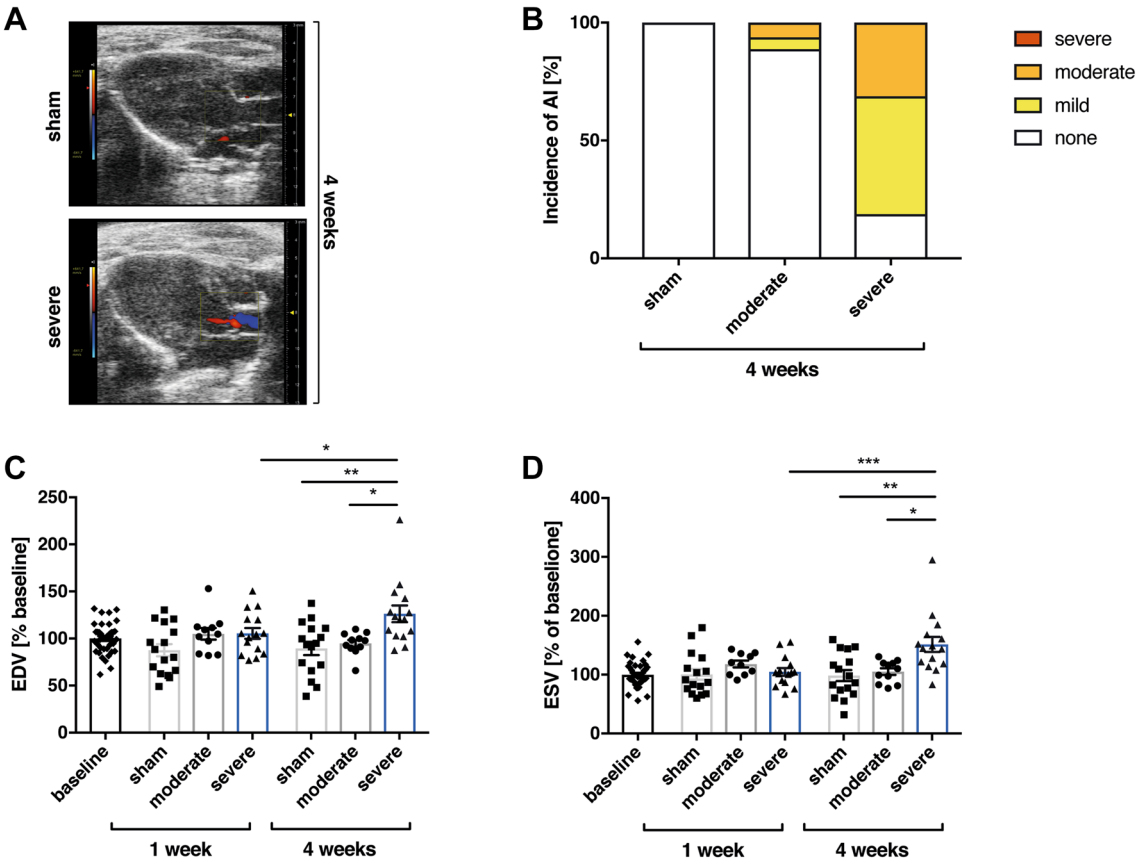

Fig. 3 Development of aortic regurgitation after wire-injury: Representative color-Doppler images in parasternal long-axis views of sham and severe-injured mice 4 weeks after surgery (**a**). Incidence of aortic valve regurgitation (**b**), left ventricular end-diastolic (**c**) and end-systolic (**d**) volumes 4 weeks after surgery in sham, moderate-injured and severe-injured mice

after 4 weeks. Upon moderate injury no changes in fractional shortening could be detected compared to sham-mice (Fig. 4b).

To analyze whether the left ventricular dysfunction is caused by volume or pressure overload, we assessed the mice for cardiac hypertrophy. Diastolic wall thickness was quantified in parasternal short- and long-axis M-Modes after moderate or severe injury. While the moderate-injured mice did not develop signs of LV hypertrophy, the severe-injured mice displayed increased thickness of the posterior wall 4 weeks after injury compared to moderate injury and sham-mice (Fig. 4c, d). These findings indicate that impaired LV function is caused by both increased volume overload following aortic regurgitation and pressure overload due to increased pressure gradient from the stenotic aortic valve.

Wire-injury leads to aortic valve thickening, inflammation and calcification

To elucidate the morphological changes after aortic valve wire-injury, the mice were sacrificed, and hearts were explanted and sectioned for histological analysis. H.E.-stained sections of severe- and morderate-injured mice revealed significant thickening of the aortic valve cusps and a greater average aortic valve area compared to sham-mice (Fig. 5a). Sham-operated mice showed thin cusps without signs of thickening.

It has been proposed that inflammation plays a critical role in the development of aortic valve stenosis in humans. To determine whether valvular inflammation is evident in this mouse model, immunofluorescence staining of CD68 was performed. Indeed, a significantly increased macrophage infiltration into the stenotic valves of moderate- and severe-injured mice compared to sham-operated mice was evident (Fig. 5b).

Additionally, fibrosis and disruption of extracellular matrix have been shown to play important roles in the development of aortic valve disease [22]. To quantify fibrosis after wire injury, Sirius-Red staining was used to visualize collagen fibers. After moderate or severe wire-injury, increased fibrosis was detectable compared to sham-mice (Fig. 5c).

Beyond cusp thickening, inflammation and fibrosis, the fourth hallmark of human AS is calcification. von Kossa staining was used to visualize valvular calcium deposits.

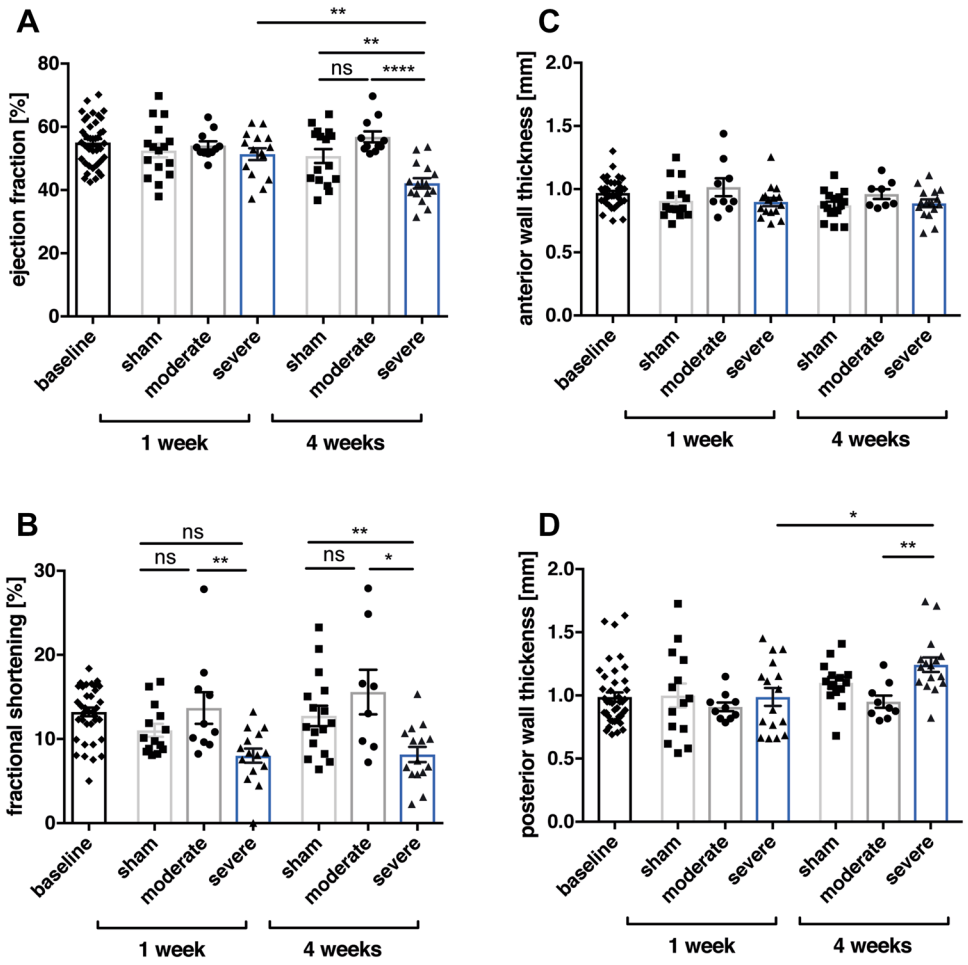

Fig. 4 Left ventricular function and hypertrophy upon wire-injury: Left ventricular ejection fraction (**a**) and fractional shortening (**b**) were measured in the parasternal long-axis view. Thickness of the anterior and posterior left ventricular walls was measured in the parasternal short-axis view (**c**)

Eight weeks after severe injury, calcium deposits were clearly evident, whereas in sham-mice and moderate-injured mice calcium was hardly detectable (Fig. 5d). Of note, no significant apoptosis could be detected at time of sacrifice (Supplemental Fig. 2).

To validate whether these histological findings mimic human disease, we examined human aortic valve specimens from patients who revived surgical valve replacement for aortic stenosis or aortic regurgitation. H.E.-stained sections form human AS-valves showed increased valve thickening with disrupted valve structure compared to non-AS valves (Supplemental Fig. 3A). Comparable to stenotic mice valves, CD68 staining also revealed macrophage infiltration into human stenotic aortic valves (Supplemental Fig. 3B).

Discussion

In this study, we successfully modified and augmented the wire-induced aortic valve injury model first described by Honda et al. Protocols were established for three different intensities of aortic valve injury resulting in mild, moderate or severe aortic valve stenosis in mice. The intensity of the injury correlated with the incidence of acute aortic regurgitation and was associated with increased mortality after surgery. Compared to moderate injury, severe injury led to greater changes in aortic valve peak velocity, mean and peak gradients. While the incidence of aortic regurgitation upon moderate injury remained low 4 weeks after surgery, severe-injured mice developed mild to moderate aortic valve regurgitation leading to mixed aortic valve disease. Pressure and volume overload were reflected by changes in LV dimension and function. While aortic regurgitation in the severe injury model was related to mild LV dilatation, these mice also displayed impaired LV ejection fraction and hypertrophic myocardium. Histological analysis verified that this mouse model mimicked all four hallmarks of human aortic valve disease including thickening of the aortic cusps, fibrosis, macrophage infiltration, and calcium deposition. The new protocols have been tested in two independent laboratories and produce consistent results (Supplemental Fig. 4).

Other models for aortic valve stenosis in mice have been published. Most of these models rely on increased serum levels of cholesterol or triglycerides combined with a

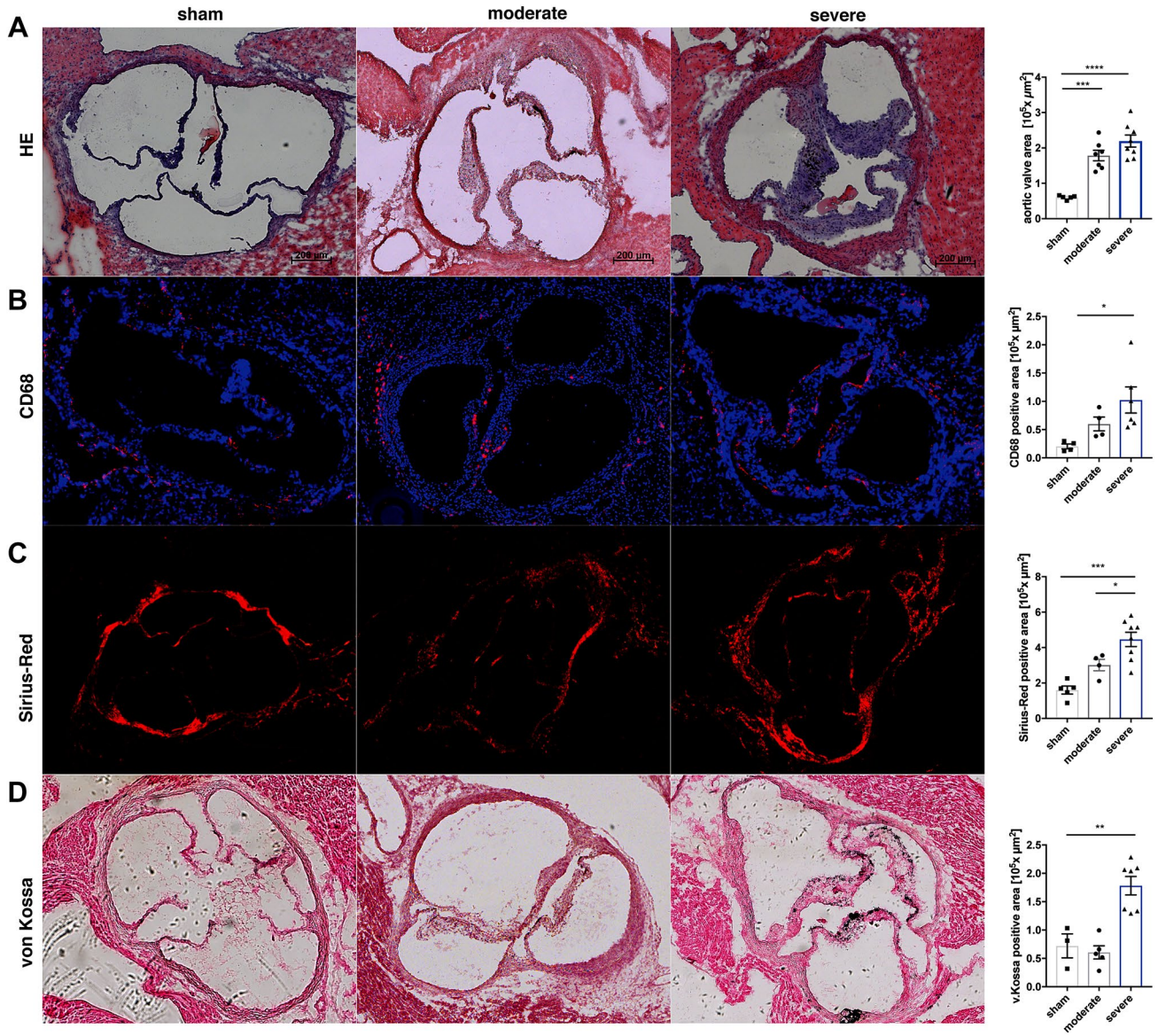

Fig. 5 Histological analysis of stenotic aortic valves: Mice were sacrificed and the hearts collected for histological analysis. Representative images and quantitative analysis of hematoxylin and eosin (HE) (**a**, aortic valve thickness), anti-CD68 immunofluorescence (**b**, macrophage infiltration), Sirius-Red (**c**, collagen I and III) and von Kossa-stained sections (**d**, calcium deposition)

genotype that supports hyperlipidemia [23]. For example, ApoE$^{-/-}$ mice develop mild AS when fed a western diet over 4–5 months. AS in this model is not restricted to cusp thickening but is also driven by cholesterol depositions in the aortic bulb [24, 25]. Certain genetic models are associated with an increased incidence of bicuspid aortic valves including eNOS$^{-/-}$ and NOTCH1$^{-/-}$ mice, and display increased blood flow velocity over the aortic valve [26, 27]. Another example is mice lacking periostin, a protein highly expressed in the endocardial cushion during embryogenesis. These mice exhibit an upregulation of delta-like-1 homolog, a negative regulator of Notch1, and display bicuspid-like aortic valves that develop aortic calcification after 10 months of age [28]. These models are valuable, as they provide insight into the role of selected proteins in aortic valve development and function. Nevertheless, these genetic models are of limited use in the testing of therapeutic interventions nor do they mimic general human pathology. For this, an AS model with a variable genetic background and applicability in wild-type mice is required.

Yet, without intervention—surgical or dietary—wild-type mice do not develop aortic valve stenosis [23]. One diet-induced model of AS in wild-type mice has been published. In this model, mice are fed with a high-fat/high-carbohydrate diet without added cholesterol over 4 months. These mice develop thickening of the aortic valve cusps

and display decreased aortic valve orifice area, but only mild to moderate AS develops [29]. The wire-induced AS injury model described by Honda et al. uniquely promotes rapid and effective development of severe AS in wild-type mice. We have expanded and improved the protocols described by Honda et al., and analyzed the concomitant incidence of aortic valve regurgitation can reliably induce consistent grade injury resulting in mild, moderate and severe AS in wild-type mice.

Additionally, AS can be induced in any mouse stain or specific transgenic animal. Thereby, the role of selected genes in the development of AS can be investigated without crossbreeding to genetical AS models. Second, the interval between surgery and AS development is gradual enough to study early disease stages but also acceptably fast for a rapid turnover and to manage therapeutic interventions. Third, the model closely mimics human disease both in relation to in vivo changes in physiology (mixed aortic valve disease and the resulting effects myocardial performance) and histopathology (cusp thickening, macrophage infiltration and calcification).

The wire-injury induced AS protocols established in this study provide a simple, reliable and consistent model of aortic valve disease in wild-type mice that closely mimics human pathology. The graded injury leading form mild to moderate or severe aortic valve stenosis can provide insight into all stages of disease development.

As with all disease models in mice, certain limitations apply. First, the acute endothelial damage via a coronary guide wire contrasts to human disease where chronic mechanical stress over decades is required for AS development. Second, mouse physiology in general differs from human physiology. Therefore, all findings using this model must ultimately be explored in a human setting.

Nevertheless, these protocols can be utilized to test hypotheses on selected mechanisms that may contribute to AS. Since no genetic alteration is needed to induce AS, it could easily be applied in transgenic mice to study specific signaling pathways. Furthermore, the efficacy of therapeutic interventions such as dietary or pharmacological treatments can be tested. Together, the wire-injury AS mouse model described here in detail may serve as an essential tool to overcome our limited understanding of AS pathology.

Acknowledgements We thank Mrs. Theresa Schmitz, Mrs. Anna Flender, Dr. Sandra Adler and Mrs. Katharina Groll for excellent technical assistance. S.N. was funded by Else-Kröner-Fresenius-Foundation of the Medical Faculty of the University of Bonn.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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