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Aortic aneurysm in diabetic mice with renovascular hypertension

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Purpose. Type 2 diabetes is the leading cause of end stage renal disease in the United States. Atherosclerotic renal artery stenosis is commonly observed in diabetic patients and impacts the rate of renal and cardiovascular disease progression. We hypothesized that renal artery stenosis contributes to bilateral renal disease in diabetics. In our original study, we found that leptin-deficient diabetic (db/db) mice subjected to RAS developed severe and bilateral renal disease, with the contralateral (uncuffed) kidney showing features reminiscent of progressive diabetic nephropathy. In non-diabetic mice (WT), the cuffed kidney developed progressive atrophy, but the contralateral kidney showed minimal histopathologic alterations. In doing these studies, we observed increased sudden death in db/db mice with RAS, but not in WT mice with RAS. The objective of this study was to characterize the aortic and cardiac phenotype of db/db mice subjected to RAS. **Methods**. We developed a murine model of renal artery stenosis by placement of a polytetrafluoroethylene cuff on the right renal artery in db/db mice. We studied 109 WT and 95 db/db mice subjected to Renal artery stenosis (RAS) or sham surgery. **Results.** The mortality rate of db/db RAS mice was about 23.5%, whereas only 1.5% deaths were observed in WT RAS mice. Interestingly, 60% of mortality in the db/db mice occurred in the first two weeks following RAS surgery. Necropsy showed massive intrathoracic hemorrhage associated with aortic dissection. Aortas from db/db RAS mice showed more smooth muscle dropout, medial disruption, and hemorrhage than aortas from WT mice with RAS. Cardiac tissue from db/db RAS mice had more fibrosis than did cardiac tissue from WT RAS mice. **Conclusions**. Db/db mice subjected to RAS are prone to develop fatal aortic dissection, which is not observed in WT mice with RAS. The db/db RAS model provides the basis for future studies directed towards defining basic mechanisms underlying the interaction of hypertension and diabetes on the development of aortic lesions.

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Aortic Aneurysm in Diabetic Mice With RenoVascular Hypertension

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ABSTRACT

Purpose. Type 2 diabetes is the leading cause of end stage renal disease in the United States. Atherosclerotic renal artery stenosis is commonly observed in diabetic patients and impacts the rate of renal and cardiovascular disease progression. We hypothesized that renal artery stenosis contributes to bilateral renal disease in diabetics. In our original study, we found that leptin-deficient diabetic (db/db) mice subjected to RAS developed severe and bilateral renal disease, with the contralateral (uncuffed) kidney showing features reminiscent of progressive diabetic nephropathy. In non-diabetic mice (WT), the cuffed kidney developed progressive atrophy, but the contralateral kidney showed minimal histopathologic alterations. In doing these studies, we observed increased sudden death in db/db mice with RAS, but not in WT mice with RAS. The objective of this study was to characterize the aortic and cardiac phenotype of db/db mice subjected to RAS.

Methods. We developed a murine model of renal artery stenosis by placement of a polytetrafluoroethylene cuff on the right renal artery in db/db mice. We studied 109 WT and 95 db/db mice subjected to Renal artery stenosis (RAS) or sham surgery.

Results. The mortality rate of db/db RAS mice was about 23.5%, whereas only 1.5% deaths were observed in WT RAS mice. Interestingly, 60% of mortality in the db/db mice occurred in the first two weeks following RAS surgery. Necropsy showed massive intrathoracic hemorrhage associated with aortic dissection. Aortas from db/db RAS mice showed more smooth muscle dropout, medial disruption, and hemorrhage than aortas from WT mice with RAS. Cardiac tissue from db/db RAS mice had more fibrosis than did cardiac tissue from WT RAS mice.

Conclusions. Db/db mice subjected to RAS are prone to develop fatal aortic dissection, which is not observed in WT mice with RAS. The db/db RAS model provides the basis for future studies

directed towards defining basic mechanisms underlying the interaction of hypertension and diabetes on the development of aortic lesions.

KEYWORDS

Diabetes, hypertension, renal artery stenosis, cardiovascular disease

INTRODUCTION

Diabetes, hypertension, and hyperlipidemia are major risk factors for the development of cardiovascular disease, the leading cause of death in the United States [1]. Diabetes is the most common cause of chronic renal disease, and is responsible for up to 50% of end stage renal disease cases in developed countries [2]. In addition to increased risk for myocardial infarction and stroke, patients with diabetes are prone to develop a diabetic cardiomyopathy, characterized by extensive fibrotic changes and cardiomyocyte hypertrophy, leading to increased myocardial stiffness and diastolic dysfunction[3, 4].

It is well recognized that hypertension is a major risk factor for both renal disease progression and cardiovascular morbidity and mortality in patients with type 2 diabetes [5]. Atherosclerotic renal artery stenosis is one of the most common causes of secondary hypertension [6]. The prevalence of renal artery stenosis approaches 7% in individuals greater than 65 years of age and is up to 45% in patients with coronary artery or aortoiliac disease[7-9]. The prevalence of renal artery stenosis varies from 17-44% in patients with hypertension and diabetes [10].

In order to study combined effects of hypertension and diabetes on renal disease progression, we have established a murine model of diabetic renovascular disease through placement of a cuff on the right renal artery of leptin deficient mice (db/db mice), which develop obesity and type 2 diabetes within the first 5 weeks of life [11]. As expected, the cuffed kidney of db/db mice, like wild-type mice, develops severe and progressive renal atrophy. Whereas the contralateral kidney of wild-type mice shows mild hyperplasia without discernable histopathologic abnormalities, severe and progressive diffuse mesangial sclerosis is identified in the contralateral kidney of db/db mice with renovascular hypertension. Histopathologic features

include progressive interstitial inflammation and fibrosis, tubular atrophy, and global glomerular sclerosis, similar to those that have been identified in progressive diabetic nephropathy [12].

Throughout the course of our studies, we found an increased incidence of sudden death in db/db mice subjected to renal artery stenosis (RAS), which was not observed in wild-type mice subjected RAS. Necropsy of mice available for analysis revealed massive hemothorax and/or hemoperitoneum, which was associated with aortic dissection.

The objective of this study was to characterize the aortic and cardiac phenotype of db/db mice subjected to RAS. We found that cardiac tissue from db/db RAS mice had more fibrosis than did cardiac tissue from WT RAS mice. Aortas from db/db RAS mice had more smooth muscle dropout, medial disruption, and hemorrhage than did aortas from WT mice with RAS. The db/db RAS model provides the basis for future studies directed towards defining basic mechanisms underlying the interaction of hypertension and diabetes on the development of aortic lesions.

METHODS

Animal Model

C57BLKS (WT) (N = 109) and C57BLKS/JLepr (db/db) (N = 95) male mice, 5-7 weeks old (Jackson Laboratory, Bar Harbor, ME) were used for the present study. Both WT and db/db mice underwent RAS or sham surgery. RAS surgery was performed by putting a cuff on right renal artery as previously described [13, 14] (N = 68 for WT and N= 64 db/db) and sham surgery were done without placing the cuff (N = 41 for WT and N= 31 db/db). All animal protocols were performed after getting approval from the Mayo Clinic Institutional Animal Care and Use Committee for appropriate experiments.

Histological and Immunohistochemical analysis

Aorta and heart tissues were fixed with 10% neutral buffered formalin and then processed for histology or immunohistochemistry using standard techniques. Histological sections of heart and aorta (5 μm thick) were stained with hematoxylin-eosin (H&E). H&E was used for scoring the aorta pathology and aortic diameter. The aortic score numbers were represented as, 0 = normal aorta; 1 = isolated small muscle dropout; 2 = multifocal small muscle dropout; 3 = hemorrhage, necrosis, dissection, thrombus, and other severe conditions. Heart sections were also stained with Masson's trichrome stain and used for the quantification of fibrosis. Width of aorta and percentage of fibrosis in heart trichrome sections were quantified at 200x magnification using an Olympus BX50 microscope (Olympus America, Melville, NY), a Micropublisher 3.3 RTV camera (Q-Imaging, Surrey, BC), and the NIS Elements Imaging Software (Nikon Instruments, Inc., Melville, NY).

For immunohistochemical analysis, sections from aortas were also stained for anti-iNOS (1:800, Abcam Inc., Cambridge, MA) and anti-CD206 (1:800, Abcam Inc., Cambridge, MA).

Real time PCR

Total RNA was isolated from the aortas using RNeasy Lipid Tissue Mini Kit (Qiagen. Valencia, CA) cDNA was made using iScript cDNA synthesis kit (BioRad, Hercules, CA). The real time PCR reaction was carried out in BioRad IQ5 instrument ((BioRad, Hercules, CA) using the CD206 and iNOS primers with SYBR green master mix (Roche Diagnostics, Deutschland GmbH).

Statistical Analysis

Data are presented as mean ± SEM. Comparisons between two groups were done using student t-test for parametric data and Mann-Whitney test for nonparametric data. For comparison across multiple groups, one-way ANOVA followed by a Turkey adjustment was used for post-hoc comparison of the measurements. P values <0.05 were considered significant. Statistical analyses were performed with Graphpad Prism 6 (GraphPad Software, La Jolla, CA).

RESULTS

db/db mice with RAS showed higher mortality rate

WT and db/db mice subjected to RAS or sham surgery were used in the present study. In our previous study, we found that db/db mice with RAS develop progressive and bilateral renal disease-the cuffed kidney shows severe interstitial fibrosis, tubular atrophy, and interstitial inflammation, whereas the contralateral kidney shows diffuse mesangial sclerosis with global glomerulosclerosis, interstitial fibrosis, tubular atrophy, and interstitial inflammation. In contrast, the cuffed kidney of WT mice showed interstitial fibrosis, inflammation, and tubular atrophy, but the contralateral kidney showed minimal histopathologic alterations. In conducting these studies, we noted that several db/db RAS mice died suddenly. Necropsy of animals for which this was possible revealed massive intrathoracic hemorrhage. We therefore extended our study to characterize the cardiovascular phenotype. The mortality of db/db mice as a function of time following RAS surgery is summarized in Table 1. A total of 204 mice were studied during this investigation. In this expanded population, the mortality of db/db RAS mice approached 23.5%, whereas only 1 mortality (1.5%) was observed in WT RAS mice. Approximately 60% of deaths in the db/db mice occurred in the two weeks following RAS surgery (Table 1).

Elevated aortic damage found in db/db RAS mice

We collected aortas for histopathologic analysis. A total of 124 aortas (N=19 WT and N=21db/db sham; N=37 WT and N=47 db/db RAS) were harvested at various time points following RAS or sham surgery and evaluated for pathological scores following H&E staining. The scoring was done using a number system by a pathologist in a random blind fashion. No damage was found in the aortas of sham mice. Aortic damage was observed in both db/db and wild type (WT) RAS mice. db/db RAS mice showed a significantly increased aortic pathological

score (mean score 1.34, p=0.0001) on the 0-3 scoring system compared to WT RAS mice (mean score= 0.405) (Figures 1 and 2).

Increase in Aortic wall width observed in damaged aortae in both WT and db/db RAS mice

The aortic width was examined at 200x magnification, from the internal elastic lamina to the adventitia. Wall thickness of aorta demonstrating histopathologic abnormalities was significantly greater than those that portrayed normal histopathology, in both db/db and WT RAS mice (p=0.002 for db/db and p=0.000 for WT) (Figure 3).

db/db RAS mice showed more cardiac fibrosis

The percentage of fibrosis was measured by computer-assisted image analysis of trichrome stained heart sections at 200x magnification in all groups (Figure 4A, B, C, D). Cardiac fibrosis was increased in both db/db and WT RAS mice compared to their sham (p = <0.0001) (Figure 4E). There was a significantly higher degree of fibrosis in db/db RAS mice compared to WT RAS mice (p = 0.045) (Figure 4E).

No significant increase in M1 and M2 markers

Real time PCR using iNOS M1 and CD206 M2 specific macrophage markers was performed. We did not observe significant difference in either M1 or M2 macrophage markers in any mice groups by histology or gene expression studies (Data not shown).

DISCUSSION

In our previous studies, we found that the contralateral kidney of db/db mice subjected to RAS developed progressive renal disease in the contralateral kidney with features of diabetic nephropathy, whereas the contralateral kidney of WT mice were mildly enlarged, but without

significant histopathologic abnormality [11]. Unexpectedly, we found that db/db mice, but not WT mice subjected to RAS had an increased prevalence of sudden death. In the animals for which it was possible to perform a necropsy, we identified massive intrathoracic hemorrhage which was associated with aortic dissection. We therefore embarked on the current study, to identify histopathologic alterations in aortas and cardiac tissue isolated from db/db and WT RAS mice. We found that db/db mice subjected to RAS developed significant aortic pathology, leading to rupture and sudden death in more than 23% of animals. Over 60% of the sudden deaths occurred in the first two weeks following RAS surgery. Histologic evaluation revealed more medial smooth muscle dropout, medial disruption, and hemorrhage in db/db RAS than WT RAS mice. Histopathologic analysis of myocardium revealed more fibrosis in db/db RAS mice than WT RAS mice.

Aortic aneurysms and dissection result from either genetic or acquired defects in the aortic wall. Since aortic aneurysms are typically asymptomatic until they rupture, it is important to better characterize the pathophysiology of aortic aneurysms and to identify patients who are at increased risk of developing these catastrophic lesions. Several animal models of aortic aneurysms have been developed to elucidate basic mechanisms underlying the development of these lesions [15].

Ang II infusion in atherosclerotic Apolipoprotein E (Apo-E) deficient mice has been employed as a model of aortic aneurysms [15-17]. In addition to increasing blood pressure, Ang II promotes influx of T cells and macrophages into the aorta and other vessels [18]. Ang II infusion promotes abdominal aortic aneurysms independent of increased blood pressure in hypercholesterolemic mice. These studies suggest that the pro-inflammatory effect of Ang II is more important than its hypertensive effect in the development of aortic aneurysms [19]. Along

these lines, the incidence of both atherosclerosis and of aortic aneurysms is significantly reduced in Apo-E deficient mice lacking CCR2, a critical receptor that directs influx of macrophages and T cells to sites of tissue injury [17].

Recent studies have defined a critical role for TGF-β signaling in the pathogenesis of aortic aneurysms and dissection. TGF- β signaling is initiated through binding of TGF- β to the type 2 receptor (TBR2), recruitment of the type 1 receptor (TBR1), followed by phosphorylation of SMAD3, recruitment of SMAD4, nuclear translocation and activation of target genes [20, 21]. Mutations in SMAD3, have been identified in up to 2% of patients with familial thoracic aneurysms leading to acute aortic dissection [22]. Patients with the Loeys-Dietz syndrome have mutations in receptors for TGF- β (TGFBR1 and TGFBR2) [23].

In mice with homozygous deletion of the Smad3 gene, angiotensin II (Ang II) infusion promotes the development of aortic aneurysms and aortic dissection. Development of aneurysms is due to Ang II mediated macrophage infiltration and upregulation of NOS2 (inducible nitric oxide synthase), matrix metalloproteinases (MMP) 2 and 9 rather than hypertension alone [24]. We did not see any significant difference in iNOS and CD206 positive macrophages.

In addition to the increased risk of developing ischemic heart disease, patients with diabetes are prone to develop diabetic cardiomyopathy, characterized by cardiac hypertrophy, myocardial fibrosis, and diastolic dysfunction [3]. Although leptin-deficient db/db mice do not develop myocardial remodeling or cardiac dysfunction, they are more susceptible to Ang II mediated hypertrophy and dysfunction [25]. Smad3 null mice crossed with leptin deficient db/db diabetic mice were protected from the development of diabetic cardiomyopathy [26]. However, db/db SMAD3 null mice showed increased mortality due to spontaneous rupture of the

ascending aorta. [26]. SMAD3 deficiency was associated with increased MMP-2 and MMP-9 activity, with no change in tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) activity. In mice fed a high fat diet or obese ob/ob mice, Ang II infusion promotes macrophage influx into the aorta and fosters the development of aortic aneurysms [27]. In our model, which employs endogenous activation of the renin-angiotensin system due to renal artery stenosis [13, 28, 29], we find that db/db mice are more susceptible to both renal and cardiovascular disease than WT mice, despite similar elevation in systolic blood pressure [11].

Although we believe that this is the first study to document aortic lesions in db/db mice subjected to renovascular hypertension, there are several limitations. First, this was a retrospective study which was not designed to identify aortic lesions. It was not possible to perform histopathologic analysis on many of the mice that died suddenly. Although an effort was made to sample grossly abnormal regions of the aorta, the focal nature of the lesions may lead to an underestimation of the degree of histologic abnormalities, including macrophage infiltration.

The murine RAS model recapitulates many of the histopathologic features of human renal artery stenosis [30]. Diabetic db/db mice subjected to RAS develop bilateral, progressive renal disease. The current studies have led to the identification of a cardiovascular phenotype characterized by myocardial fibrosis and degenerative alterations within the aorta which predispose the development of aortic dissection. Future studies will determine whether the diabetic phenotype interacts with the pro-inflammatory state driven by elevated Ang II levels promotes NOS2 generation or induces MMP activity, which have been implicated in other murine models of aortic aneurysms.

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FIGURE LEGENDS

Table 1. Number of mortality observed in WT and db/db mice as function of time. db/db RAS mice showed the highest mortality.

Figure 1. Aortic pathology. Representative images of aorta illustrating semiquantitative histologic assessment scores. A) shows normal aorta (score of 0. B) Focal myocyte dropout (Score of 1). C) Multifocal myocyte dropout (Score of 2). D) Medial disruption and hemorrhage (Score of 3).

Figure 2. db/db mice showed higher mean pathology score. Mean pathology score of db/db and WT mice subjected to RAS surgery. The aortic pathology score in db/db RAS mice was significantly more compared to WT RAS mice ($p = 0.0001$).

Figure 3. Abnormal aorta showed increased overall wall thickness. Mean aortic medial thickness was greater in aortas with histopathologic scores of 1, 2, or 3 versus a score of 0 in both WT and db/db mice ($p = 0.00$).

Figure 4. Increased cardiac fibrosis in db/db RAS mice. Myocardial fibrosis was assessed by quantitative image analysis of trichrome stained sections at 200x magnification obtained from dbc sham (A), db sham (B), dbc RAS, and db RAS (D) mice. (E). The mean percentage of fibrosis in db/db and WT sham and RAS mice. Both WT and db/db showed increase % fibrosis

following RAS ($p = 0.000$ compared to their respective sham). db/db RAS mice had significantly more fibrosis ($p = 0.04$)

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389 **Table 1.**

	Days Following Surgery												
Mouse strain-surgery	**1**	**3**	**4**	**5**	**7**	**9**	**14**	**28**	**40**	**61**	**90**	**104**	**117**
db/db-RAS	1	2	1	2	2	0	1	1	1	1	1	1	1
db/db- Sham	0	0	0	0	0	0	0	0	0	0	0	0	0
WT-RAS	0	0	0	0	0	1	0	0	0	0	0	0	0
WT-Sham	0	0	0	0	0	0	0	0	0	0	0	0	0

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399 **Figure 1.**

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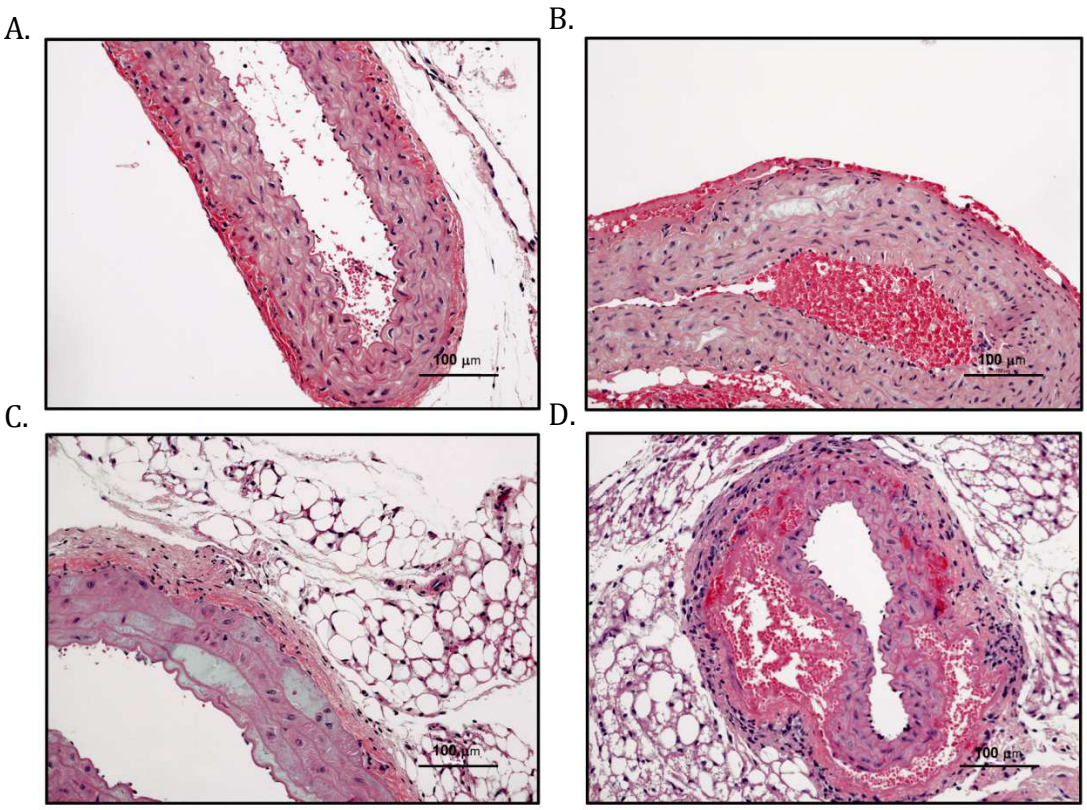

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404 **Figure 2.**

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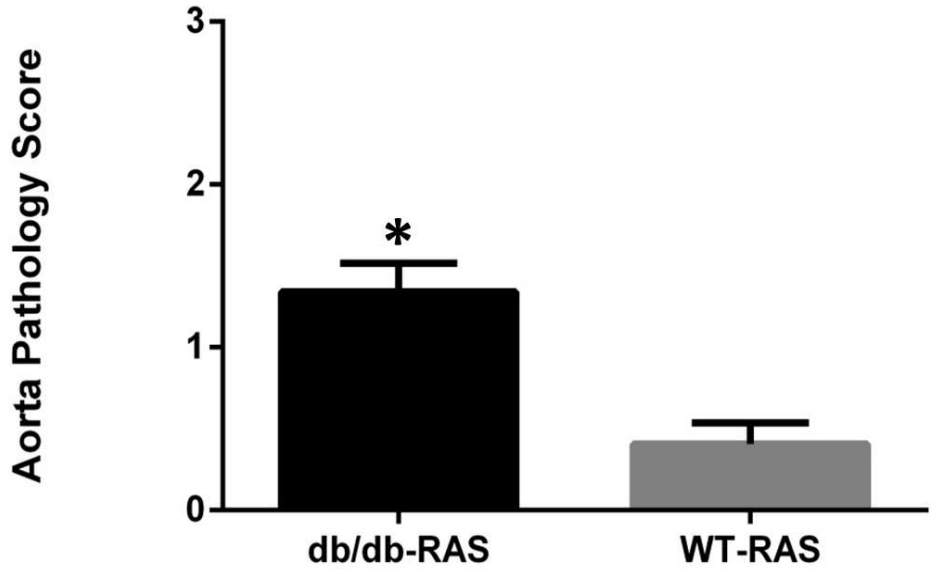

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Figure 3

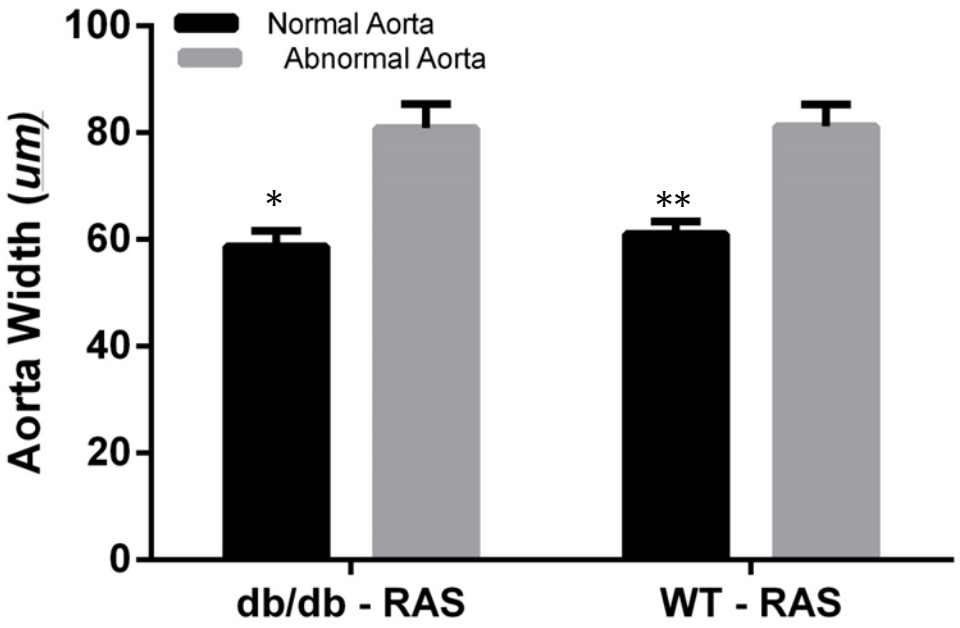
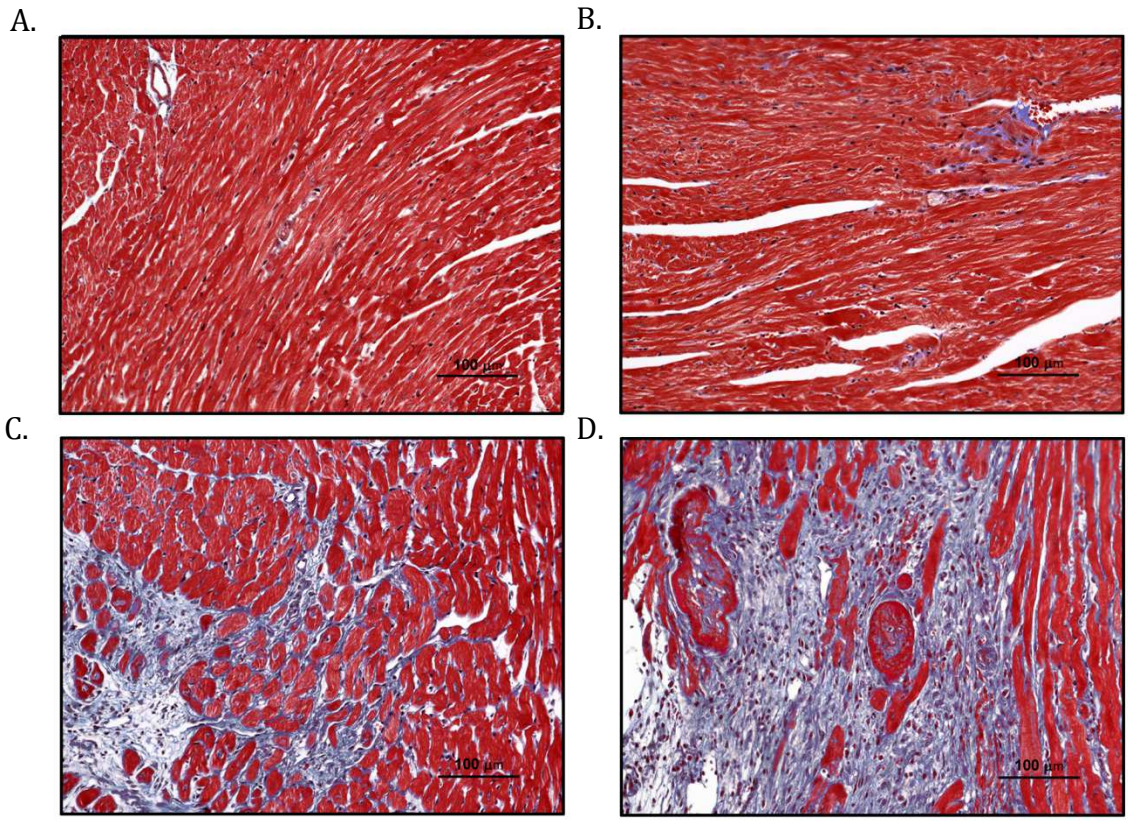
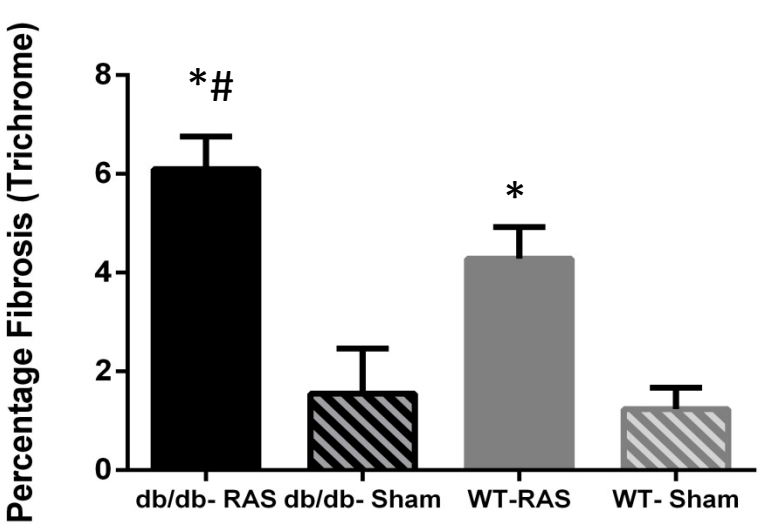


Figure 4.





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