Aortic aneurysm in diabetic mice with renovascular hypertension

Sonu Kashyap, Sean Engel, Yousif Al-Saiegh, Asarn Wongjarupong, Joseph P Grande

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.

1   **Aortic Aneurysm in Diabetic Mice With RenoVascular Hypertension**

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24 **ABSTRACT**

25 **Purpose.** Type 2 diabetes is the leading cause of end stage renal disease in the United States.

26 Atherosclerotic renal artery stenosis is commonly observed in diabetic patients and impacts the

27 rate of renal and cardiovascular disease progression. We hypothesized that renal artery stenosis

28 contributes to bilateral renal disease in diabetics. In our original study, we found that leptin-

29 deficient diabetic (db/db) mice subjected to RAS developed severe and bilateral renal disease,

30 with the contralateral (uncuffed) kidney showing features reminiscent of progressive diabetic

31 nephropathy. In non-diabetic mice (WT), the cuffed kidney developed progressive atrophy, but

32 the contralateral kidney showed minimal histopathologic alterations. In doing these studies, we

33 observed increased sudden death in db/db mice with RAS, but not in WT mice with RAS. The

34 objective of this study was to characterize the aortic and cardiac phenotype of db/db mice

35 subjected to RAS.

36 **Methods**. We developed a murine model of renal artery stenosis by placement of a

37 polytetrafluoroethylene cuff on the right renal artery in db/db mice. We studied 109 WT and 95

38 db/db mice subjected to Renal artery stenosis (RAS) or sham surgery.

39 **Results.** The mortality rate of db/db RAS mice was about 23.5%, whereas only 1.5% deaths

40 were observed in WT RAS mice. Interestingly, 60% of mortality in the db/db mice occurred in

41 the first two weeks following RAS surgery. Necropsy showed massive intrathoracic hemorrhage

42 associated with aortic dissection. Aortas from db/db RAS mice showed more smooth muscle

43 dropout, medial disruption, and hemorrhage than aortas from WT mice with RAS. Cardiac tissue

44 from db/db RAS mice had more fibrosis than did cardiac tissue from WT RAS mice.

45 **Conclusions**. Db/db mice subjected to RAS are prone to develop fatal aortic dissection, which is

46 not observed in WT mice with RAS. The db/db RAS model provides the basis for future studies

47   directed towards defining basic mechanisms underlying the interaction of hypertension and

48   diabetes on the development of aortic lesions.

49   **KEYWORDS**

50   Diabetes, hypertension, renal artery stenosis, cardiovascular disease

51
52 **INTRODUCTION**

53   Diabetes, hypertension, and hyperlipidemia are major risk factors for the development of

54 cardiovascular disease, the leading cause of death in the United States [1]. Diabetes is the most

55 common cause of chronic renal disease, and is responsible for up to 50% of end stage renal

56 disease cases in developed countries [2]. In addition to increased risk for myocardial infarction

57 and stroke, patients with diabetes are prone to develop a diabetic cardiomyopathy, characterized

58 by extensive fibrotic changes and cardiomyocyte hypertrophy, leading to increased myocardial

59 stiffness and diastolic dysfunction[3, 4].

60   It is well recognized that hypertension is a major risk factor for both renal disease

61 progression and cardiovascular morbidity and mortality in patients with type 2 diabetes [5].

62 Atherosclerotic renal artery stenosis is one of the most common causes of secondary

63 hypertension [6]. The prevalence of renal artery stenosis approaches 7% in individuals greater

64 than 65 years of age and is up to 45% in patients with coronary artery or aortoiliac disease[7-9].

65 The prevalence of renal artery stenosis varies from 17-44% in patients with hypertension and

66 diabetes [10].

67   In order to study combined effects of hypertension and diabetes on renal disease

68 progression, we have established a murine model of diabetic renovascular disease through

69 placement of a cuff on the right renal artery of leptin deficient mice (db/db mice), which develop

70 obesity and type 2 diabetes within the first 5 weeks of life [11]. As expected, the cuffed kidney

71 of db/db mice, like wild-type mice, develops severe and progressive renal atrophy. Whereas the

72 contralateral kidney of wild-type mice shows mild hyperplasia without discernable

73 histopathologic abnormalities, severe and progressive diffuse mesangial sclerosis is identified in

74 the contralateral kidney of db/db mice with renovascular hypertension. Histopathologic features

75   include progressive interstitial inflammation and fibrosis, tubular atrophy, and global glomerular

76   sclerosis, similar to those that have been identified in progressive diabetic nephropathy [12].

77   Throughout the course of our studies, we found an increased incidence of sudden death in

78   db/db mice subjected to renal artery stenosis (RAS), which was not observed in wild-type mice

79   subjected RAS. Necropsy of mice available for analysis revealed massive hemothorax and/or

80   hemoperitoneum, which was associated with aortic dissection.

81   The objective of this study was to characterize the aortic and cardiac phenotype of db/db

82   mice subjected to RAS. We found that cardiac tissue from db/db RAS mice had more fibrosis

83   than did cardiac tissue from WT RAS mice. Aortas from db/db RAS mice had more smooth

84   muscle dropout, medial disruption, and hemorrhage than did aortas from WT mice with RAS.

85   The db/db RAS model provides the basis for future studies directed towards defining basic

86   mechanisms underlying the interaction of hypertension and diabetes on the development of aortic

87   lesions.

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91 **METHODS**

92 *Animal Model*

93   C57BLKS (WT) (N = 109) and C57BLKS/JLepr (db/db) (N = 95) male mice, 5-7 weeks

94 old (Jackson Laboratory, Bar Harbor, ME) were used for the present study. Both WT and db/db

95 mice underwent RAS or sham surgery. RAS surgery was performed by putting a cuff on right

96 renal artery as previously described [13, 14] (N = 68 for WT and N= 64 db/db) and sham surgery

97 were done without placing the cuff (N = 41 for WT and N= 31 db/db). All animal protocols were

98 performed after getting approval from the Mayo Clinic Institutional Animal Care and Use

99 Committee for appropriate experiments.

100

101 *Histological and Immunohistochemical analysis*

102   Aorta and heart tissues were fixed with 10% neutral buffered formalin and then processed

103 for histology or immunohistochemistry using standard techniques. Histological sections of heart

104 and aorta (5 μm thick) were stained with hematoxylin-eosin (H&E). H&E was used for scoring

105 the aorta pathology and aortic diameter. The aortic score numbers were represented as, 0 =

106 normal aorta; 1 = isolated small muscle dropout; 2 = multifocal small muscle dropout; 3 =

107 hemorrhage, necrosis, dissection, thrombus, and other severe conditions. Heart sections were

108 also stained with Masson's trichrome stain and used for the quantification of fibrosis. Width of

109 aorta and percentage of fibrosis in heart trichrome sections were quantified at 200x

110 magnification using an Olympus BX50 microscope (Olympus America, Melville, NY), a

111 Micropublisher 3.3 RTV camera (Q-Imaging, Surrey, BC), and the NIS Elements Imaging

112 Software (Nikon Instruments, Inc., Melville, NY).

113   For immunohistochemical analysis, sections from aortas were also stained for anti-iNOS

114   (1:800, Abcam Inc., Cambridge, MA) and anti-CD206 (1:800, Abcam Inc., Cambridge, MA).

115   ***Real time PCR***

116   Total RNA was isolated from the aortas using RNeasy Lipid Tissue Mini Kit (Qiagen.

117   Valencia, CA) cDNA was made using iScript cDNA synthesis kit (BioRad, Hercules, CA). The

118   real time PCR reaction was carried out in BioRad IQ5 instrument ((BioRad, Hercules, CA)

119   using the CD206 and iNOS primers with SYBR green master mix (Roche Diagnostics,

120   Deutschland GmbH).

121

122   ***Statistical Analysis***

123   Data are presented as mean ± SEM. Comparisons between two groups were done using

124   student t-test for parametric data and Mann-Whitney test for nonparametric data. For comparison

125   across multiple groups, one-way ANOVA followed by a Turkey adjustment was used for post-

126   hoc comparison of the measurements. P values <0.05 were considered significant. Statistical

127   analyses were performed with Graphpad Prism 6 (GraphPad Software, La Jolla, CA).

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129
130 **RESULTS**

131 *db/db mice with RAS showed higher mortality rate*

132   WT and db/db mice subjected to RAS or sham surgery were used in the present study. In

133 our previous study, we found that db/db mice with RAS develop progressive and bilateral renal

134 disease-the cuffed kidney shows severe interstitial fibrosis, tubular atrophy, and interstitial

135 inflammation, whereas the contralateral kidney shows diffuse mesangial sclerosis with global

136 glomerulosclerosis, interstitial fibrosis, tubular atrophy, and interstitial inflammation. In contrast,

137 the cuffed kidney of WT mice showed interstitial fibrosis, inflammation, and tubular atrophy, but

138 the contralateral kidney showed minimal histopathologic alterations. In conducting these studies,

139 we noted that several db/db RAS mice died suddenly. Necropsy of animals for which this was

140 possible revealed massive intrathoracic hemorrhage. We therefore extended our study to

141 characterize the cardiovascular phenotype. The mortality of db/db mice as a function of time

142 following RAS surgery is summarized in Table 1. A total of 204 mice were studied during this

143 investigation. In this expanded population, the mortality of db/db RAS mice approached 23.5%,

144 whereas only 1 mortality (1.5%) was observed in WT RAS mice. Approximately 60% of deaths

145 in the db/db mice occurred in the two weeks following RAS surgery (Table 1).

146 *Elevated aortic damage found in db/db RAS mice*

147   We collected aortas for histopathologic analysis. A total of 124 aortas (N=19 WT and

148 N=21db/db sham; N=37 WT and N=47 db/db RAS) were harvested at various time points

149 following RAS or sham surgery and evaluated for pathological scores following H&E staining.

150 The scoring was done using a number system by a pathologist in a random blind fashion. No

151 damage was found in the aortas of sham mice. Aortic damage was observed in both db/db and

152 wild type (WT) RAS mice. db/db RAS mice showed a significantly increased aortic pathological

153   score (mean score 1.34, p=0.0001) on the 0-3 scoring system compared to WT RAS mice (mean

154   score= 0.405) (Figures 1 and 2).

155

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

157   The aortic width was examined at 200x magnification, from the internal elastic lamina to

158   the adventitia. Wall thickness of aorta demonstrating histopathologic abnormalities was

159   significantly greater than those that portrayed normal histopathology, in both db/db and WT RAS

160   mice (p=0.002 for db/db and p=0.000 for WT) (Figure 3).

161   ***db/db RAS mice showed more cardiac fibrosis***

162   The percentage of fibrosis was measured by computer-assisted image analysis of

163   trichrome stained heart sections at 200x magnification in all groups (Figure 4A, B, C, D).

164   Cardiac fibrosis was increased in both db/db and WT RAS mice compared to their sham (p =

165   <0.0001) (Figure 4E). There was a significantly higher degree of fibrosis in db/db RAS mice

166   compared to WT RAS mice (p = 0.045) (Figure 4E).

167   ***No significant increase in M1 and M2 markers***

168   Real time PCR using iNOS M1 and CD206 M2 specific macrophage markers was

169   performed. We did not observe significant difference in either M1 or M2 macrophage markers in

170   any mice groups by histology or gene expression studies (Data not shown).

171

172   **DISCUSSION**

173   In our previous studies, we found that the contralateral kidney of db/db mice subjected to

174   RAS developed progressive renal disease in the contralateral kidney with features of diabetic

175   nephropathy, whereas the contralateral kidney of WT mice were mildly enlarged, but without

176 significant histopathologic abnormality [11]. Unexpectedly, we found that db/db mice, but not

177 WT mice subjected to RAS had an increased prevalence of sudden death. In the animals for

178 which it was possible to perform a necropsy, we identified massive intrathoracic hemorrhage

179 which was associated with aortic dissection. We therefore embarked on the current study, to

180 identify histopathologic alterations in aortas and cardiac tissue isolated from db/db and WT RAS

181 mice. We found that db/db mice subjected to RAS developed significant aortic pathology,

182 leading to rupture and sudden death in more than 23% of animals. Over 60% of the sudden

183 deaths occurred in the first two weeks following RAS surgery. Histologic evaluation revealed

184 more medial smooth muscle dropout, medial disruption, and hemorrhage in db/db RAS than WT

185 RAS mice. Histopathologic analysis of myocardium revealed more fibrosis in db/db RAS mice

186 than WT RAS mice.

187   Aortic aneurysms and dissection result from either genetic or acquired defects in the

188 aortic wall. Since aortic aneurysms are typically asymptomatic until they rupture, it is important

189 to better characterize the pathophysiology of aortic aneurysms and to identify patients who are at

190 increased risk of developing these catastrophic lesions. Several animal models of aortic

191 aneurysms have been developed to elucidate basic mechanisms underlying the development of

192 these lesions [15].

193   Ang II infusion in atherosclerotic Apolipoprotein E (Apo-E) deficient mice has been

194 employed as a model of aortic aneurysms [15-17]. In addition to increasing blood pressure, Ang

195 II promotes influx of T cells and macrophages into the aorta and other vessels [18]. Ang II

196 infusion promotes abdominal aortic aneurysms independent of increased blood pressure in

197 hypercholesterolemic mice. These studies suggest that the pro-inflammatory effect of Ang II is

198 more important than its hypertensive effect in the development of aortic aneurysms [19]. Along

199 these lines, the incidence of both atherosclerosis and of aortic aneurysms is significantly reduced

200 in Apo-E deficient mice lacking CCR2, a critical receptor that directs influx of macrophages and

201 T cells to sites of tissue injury [17].

202   Recent studies have defined a critical role for TGF-β signaling in the pathogenesis of

203 aortic aneurysms and dissection. TGF- β signaling is initiated through binding of TGF- β to the

204 type 2 receptor (TBR2), recruitment of the type 1 receptor (TBR1), followed by phosphorylation

205 of SMAD3, recruitment of SMAD4, nuclear translocation and activation of target genes [20, 21].

206 Mutations in SMAD3, have been identified in up to 2% of patients with familial thoracic

207 aneurysms leading to acute aortic dissection [22]. Patients with the Loeys-Dietz syndrome have

208 mutations in receptors for TGF- β (TGFBR1 and TGFBR2) [23].

209 In mice with homozygous deletion of the Smad3 gene, angiotensin II (Ang II) infusion promotes

210 the development of aortic aneurysms and aortic dissection. Development of aneurysms is due to

211 Ang II mediated macrophage infiltration and upregulation of NOS2 (inducible nitric oxide

212 synthase), matrix metalloproteinases (MMP) 2 and 9 rather than hypertension alone [24]. We did

213 not see any significant difference in iNOS and CD206 positive macrophages.

214   In addition to the increased risk of developing ischemic heart disease, patients with

215 diabetes are prone to develop diabetic cardiomyopathy, characterized by cardiac hypertrophy,

216 myocardial fibrosis, and diastolic dysfunction [3]. Although leptin-deficient db/db mice do not

217 develop myocardial remodeling or cardiac dysfunction, they are more susceptible to Ang II

218 mediated hypertrophy and dysfunction [25]. Smad3 null mice crossed with leptin deficient db/db

219 diabetic mice were protected from the development of diabetic cardiomyopathy [26]. However,

220 db/db SMAD3 null mice showed increased mortality due to spontaneous rupture of the

221   ascending aorta. [26]. SMAD3 deficiency was associated with increased MMP-2 and MMP-9

222   activity, with no change in tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) activity.

223   In mice fed a high fat diet or obese ob/ob mice, Ang II infusion promotes macrophage influx into

224   the aorta and fosters the development of aortic aneurysms [27]. In our model, which employs

225   endogenous activation of the renin-angiotensin system due to renal artery stenosis [13, 28, 29],

226   we find that db/db mice are more susceptible to both renal and cardiovascular disease than WT

227   mice, despite similar elevation in systolic blood pressure [11].

228   Although we believe that this is the first study to document aortic lesions in db/db mice subjected

229   to renovascular hypertension, there are several limitations. First, this was a retrospective study

230   which was not designed to identify aortic lesions. It was not possible to perform histopathologic

231   analysis on many of the mice that died suddenly. Although an effort was made to sample grossly

232   abnormal regions of the aorta, the focal nature of the lesions may lead to an underestimation of

233   the degree of histologic abnormalities, including macrophage infiltration.

234   The murine RAS model recapitulates many of the histopathologic features of human

235   renal artery stenosis [30]. Diabetic db/db mice subjected to RAS develop bilateral, progressive

236   renal disease. The current studies have led to the identification of a cardiovascular phenotype

237   characterized by myocardial fibrosis and degenerative alterations within the aorta which

238   predispose the development of aortic dissection. Future studies will determine whether the

239   diabetic phenotype interacts with the pro-inflammatory state driven by elevated Ang II levels

240   promotes NOS2 generation or induces MMP activity, which have been implicated in other

241   murine models of aortic aneurysms.

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251

252   **FIGURE LEGENDS**

253   **Table 1.** Number of mortality observed in WT and db/db mice as function of time. db/db RAS

254   mice showed the highest mortality.

255

256   **Figure 1. Aortic pathology.** Representative images of aorta illustrating semiquantitative

257   histologic assessment scores. A) shows normal aorta (score of 0. B) Focal myocyte dropout

258   (Score of 1). C) Multifocal myocyte dropout (Score of 2). D) Medial disruption and hemorrhage

259   (Score of 3).

260

261   **Figure 2. db/db mice showed higher mean pathology score.** Mean pathology score of db/db

262   and WT mice subjected to RAS surgery. The aortic pathology score in db/db RAS mice was

263   significantly more compared to WT RAS mice (p = 0.0001).

264

265   **Figure 3. Abnormal aorta showed increased overall wall thickness.** Mean aortic medial

266   thickness was greater in aortas with histopathologic scores of 1, 2, or 3 versus a score of 0 in

267   both WT and db/db mice (p = 0.00).

268

269   **Figure 4. Increased cardiac fibrosis in db/db RAS mice.** Myocardial fibrosis was assessed by

270   quantitative image analysis of trichrome stained sections at 200x magnification obtained from

271   dbc sham (A), db sham (B), dbc RAS, and db RAS (D) mice. (E). The mean percentage of

272   fibrosis in db/db and WT sham and RAS mice. Both WT and db/db showed increase % fibrosis

273   following RAS (p =0.000 compared to their respective sham). db/db RAS mice had significantly

274   more fibrosis (p=0.04)

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383   **nephropathy secondary to atherosclerotic renal artery stenosis: clinical and**

384   **histopathological correlates**. *Nephrol Dial Transplant* 2010.

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389   **Table 1.**

Mouse strain-surgery	Days Following 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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B.

C.

D.

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404 **Figure 2.**

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411   **Figure 3**

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424   **Figure 4.**

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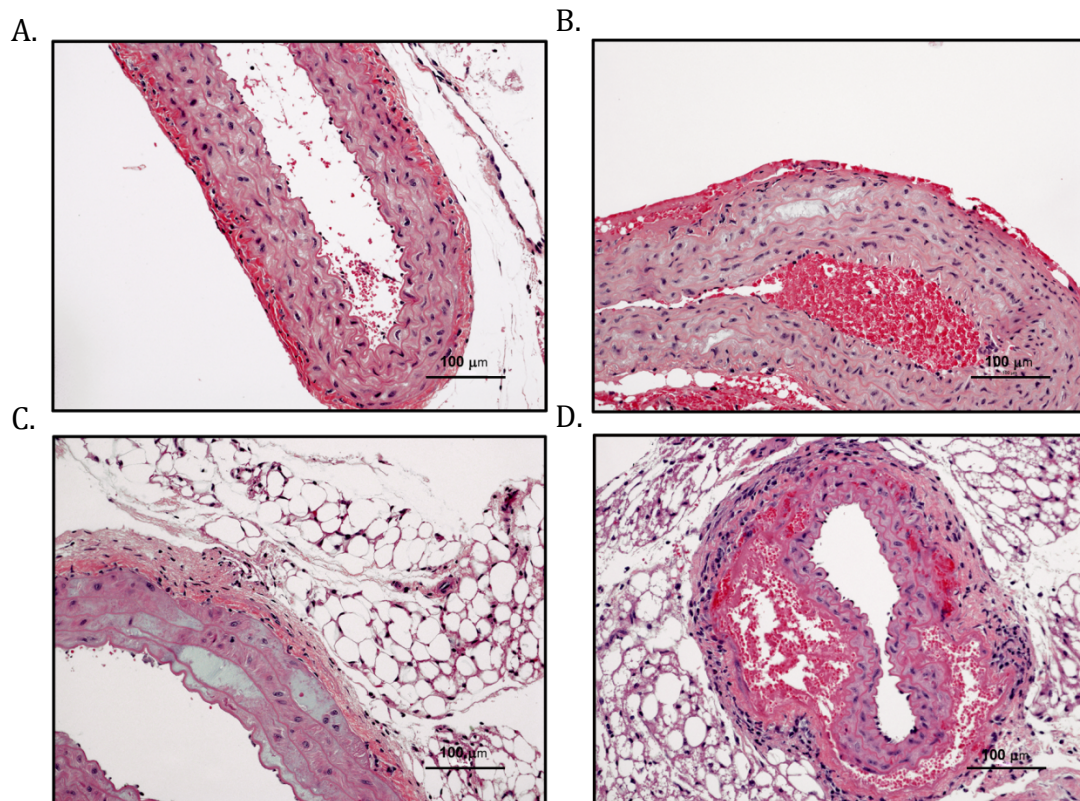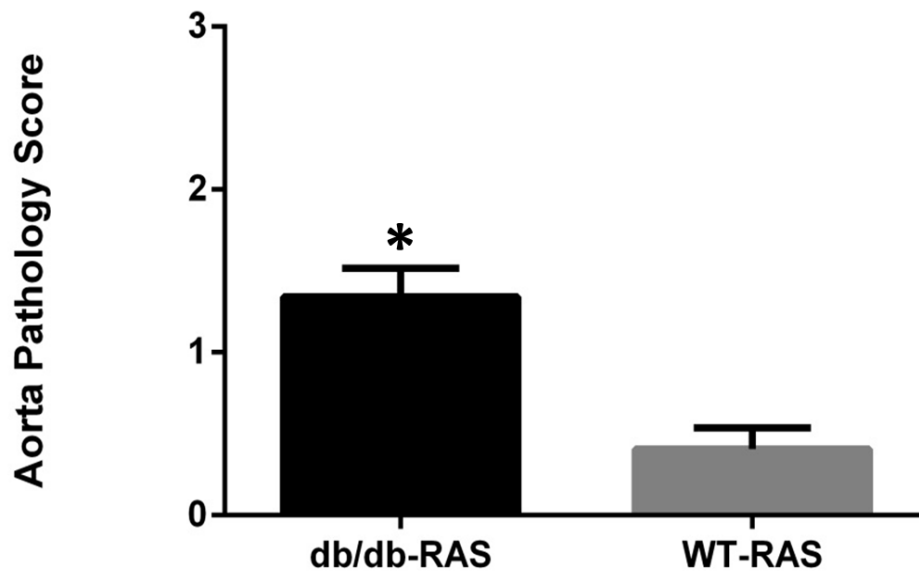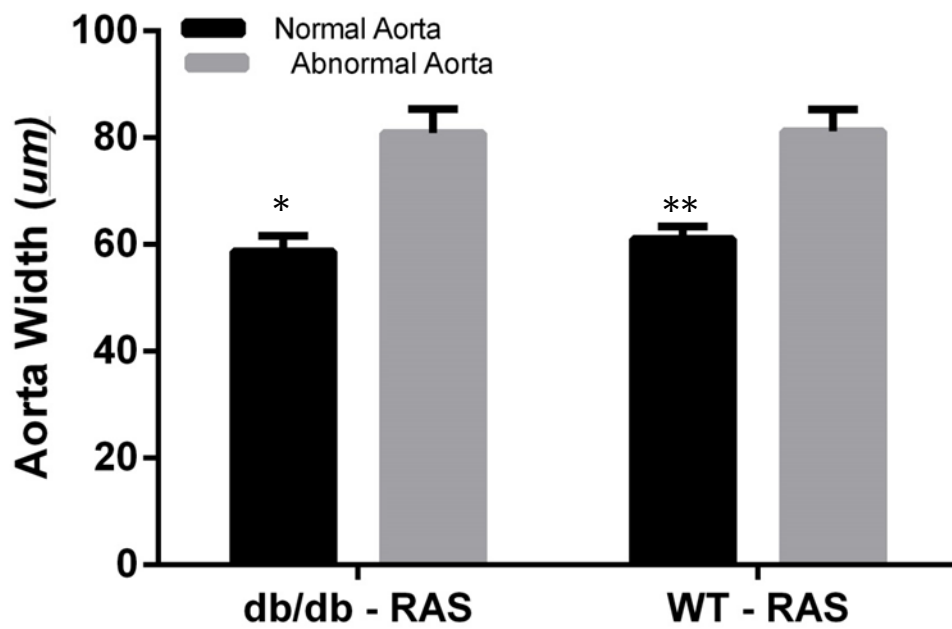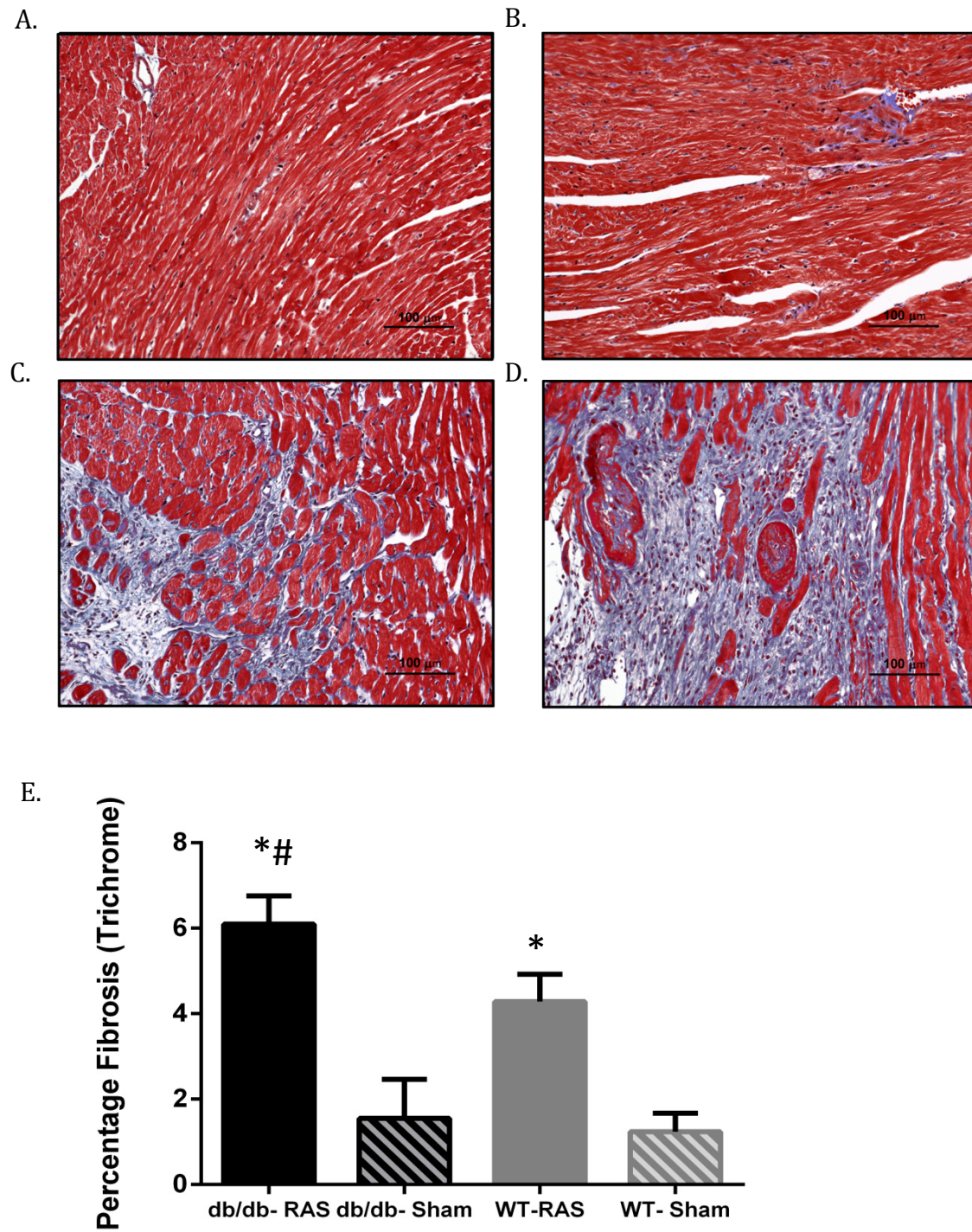
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