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TIMP-2 mutant decreases MMP-2 activity and augments pressure overload induced LV dysfunction and heart failure

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Abstract

Pressure overload induces cardiac extracellular matrix (ECM) remodelling and results in heart failure. ECM remodelling by matrix metalloproteinases (MMPs) is primarily regulated by their target inhibitors, tissue inhibitor of matrix metalloproteinases (TIMPs). It is known that TIMP-2 is highly expressed in myocardium and is required for cell surface activation of pro-MMP-2. We and others have reported that imbalance between angiogenic growth factors and anti-angiogenic factors results in transition from compensatory cardiac hypertrophy to heart failure. We previously reported the pro-angiogenic role of MMP-2 in cardiac compensation, however, the specific role of TIMP-2 during pressure overload is yet unclear. We hypothesize that genetic ablation of TIMP-2 exacerbates the adverse cardiac matrix remodelling due to lack of pro-angiogenic MMP-2 and increase in anti-angiogenic factors during pressure overload stress and results in severe heart failure. To verify this, ascending aortic banding (AB) was created to mimic pressure overload, in wild type C57BL6/J and TIMP-2^{-/-} (model of MMP-2 deficiency) mice. Left ventricular (LV) function assessed by echocardiography and pressure-volume loop studies showed severe LV dysfunction in TIMP-2^{-/-} AB mice compared to controls. Expression of MMP-2, vascular endothelial growth factor (VEGF) was decreased and expression of MMP-9, anti-angiogenic factors endostatin and angiostatin was increased in TIMP-2^{-/-} AB mice compared with wild type AB mice. Connexins (Cx) are the gap junction proteins that are widely present in the myocardium and play an important role in endothelial-myocyte coupling. Our results showed that expression of Cx 37 and 43 was decreased in TIMP-2^{-/-} AB mice compared with corresponding wild type controls. These results suggest that genetic ablation of TIMP-2 decrease the expression of pro-angiogenic MMP-2, VEGF and increases anti-angiogenic factors that results in exacerbated abnormal ventricular remodelling leading to severe heart failure.

Keywords

Angiogenesis; connexins; heart failure; matrix remodelling; pressure overload; TIMP-2

Introduction

We and others have shown that pressure overload on the heart leads to initial compensatory hypertrophy and on sustained overload, resulting in de-compensatory heart failure (Frey & Olson, 2003; Givvimani *et al.*, 2010, 2011a). During compensatory hypertrophy, expression

of pro-angiogenic MMP-2 along with angiogenic factor VEGF (vascular endothelial growth factor) was increased and during de-compensatory heart failure, expression of MMP-9, TIMP-3 and anti-angiogenic factors like endostatin and angiostatin was increased (Givvimani *et al.*, 2010; Sodha *et al.*, 2009). Previous studies report that MMP-2 plays a significant role during heart valve development (Alexander *et al.*, 1997). It was also reported that MMP-2 knockout mice have various cardiac anomalies during embryogenesis (Robbins *et al.*, 1999).

Hypertension or pressure overload on the heart is considered as one of the important risk factors of heart failure. Increased after-load leads to left ventricular remodelling leading to cardiac dysfunction and heart failure (Toischer *et al.*, 2010). Extracellular matrix remodelling by matrix metalloproteinases (MMPs) during pressure overload is mainly regulated by their tissue inhibitors, tissue inhibitor of matrix metalloproteinases (TIMPs) (Kassiri & Khokha, 2005; Spinale, 2007). There are four TIMPs identified so far, of which TIMP-2 and TIMP-3 are highly expressed in the myocardium (Nuttall *et al.*, 2004). TIMP-2 is unique in that it has dual actions of activating pro-MMP-2 and also inhibition of active MMP-2 (Strongin *et al.*, 1995). TIMP-2 is highly important for activating pro-MMP-2 to active MMP-2. The association of TIMP-2 to cardiac dysfunction and fibrosis during pressure overload has been reported previously (Heymans *et al.*, 2005). The decreased expression of MMP-2 results in decreased angiogenesis promoting to heart failure (Givvimani *et al.*, 2010, 2011a). Though TIMP-2 activates MMP-2, excess TIMP-2 inhibits MMP-2 (Brew & Nagase, 2010; Butler *et al.*, 1998; Strongin *et al.*, 1995). Membrane type 1 MMP (MT1-MMP), also called MMP-14 forms a tri-molecular complex with MMP-2 and TIMP-2 during activation of pro-MMP (Strongin *et al.*, 1995). Interestingly TIMP-2 also has an inhibitory action on MT1-MMP (Sato *et al.*, 1996). Though recent literature reported the role of TIMP-2 in pressure overload heart disease, we are trying to pursue this study in a different aspect of the disease mechanism (Kandalam, 2011).

Connexins are the main gap junction proteins that play an important role during cardiac contraction. Previously we have reported that decreased connexin expression plays an important role in disrupting endothelial-cardiomyocyte coupling and inducing arrhythmogenicity in heart during hyperhomocysteinemia and heart failure (Givvimani *et al.*, 2011b). Recent study also reported that reduced connexin 43 expression enhances fibrosis deposition in pressure overload mice models (Jansen *et al.*, 2012). Accordingly, we studied connexin expression in TIMP-2^{-/-} aortic banding mice in our current study.

We and others have shown that during pressure overload hypertrophy, angiogenic imbalance leads to transition from compensatory hypertrophy to de-compensatory heart failure. We also showed that MMP-2 is pro-angiogenic and induce the release of angiogenic growth factors like vascular endothelial growth factor (VEGF). Accordingly, we hypothesize that genetic ablation of TIMP-2 exacerbates the adverse cardiac matrix remodelling due to lack of pro-angiogenic MMP-2 and increase in anti-angiogenic factors during pressure overload stress and results in severe heart failure.

Material and methods

Animals

Male wild type mice (WT, C57BL/6J) and TIMP-2^{-/-} mice aged 12 weeks were procured from Jackson Laboratories (Bar Harbor, ME) and housed in the animal care facility at University of Louisville with access to standard chow and water. Previous literature suggests that the control or background strain for TIMP-2 KO mice is C57BL/6J and there is deficient activation of pro-MMP-2 in these mice (Wang *et al.*, 2000). Ascending aortic banding was created in mice of 12 weeks age with an approximate weight of 23–25 grams.

Sham group of animals were treated with similar protocol except for aortic banding. Animals were followed for 8 weeks period by which time they attain heart failure status. After the study period, animals were euthanized in accordance with National Institute of Health Guidelines for animal research and were reviewed and approved by the Institute Animal Care and use Committee of University of Louisville (IACUC # 07134).

Pressure overload animal model

Ascending aortic banding was done as described previously (Givvimani *et al.*, 2010; 2011a). Briefly, under sodium pentobarbital anaesthesia, animals were intubated and ventilated with a Harvard mini ventilator. Body temperature was maintained with a heating pad. Under sterile surgical environment thorax was opened by left parasternal thoracotomy and ascending aorta was dissected and separated from the adjacent structures. Ascending aorta was ligated with 6-0 silk by placing the 26 g needle on the aorta for optimum constriction. Needle was quickly removed to keep the constricted aorta patent. Wound was closed in layers using 6-0 vicryl for the subcutaneous tissues and 5-0 silk to the skin. The mortality rate was less than 20% with the surgery. All animals were given post-operative analgesia with intraperitoneal injection of Ketofen, 5 mg/kg body weight. Sham group of animals underwent similar procedure except the aortic constriction. By creating pressure overload using ascending aortic banding model, heart failure develops by 8 weeks. Our previous experiments showed the pathophysiological and histological changes associated with heart failure are achieved by 8 weeks (Givvimani *et al.*, 2010; 2011a).

Antibodies and reagents

The following primary antibodies were used for western blot and immunohistochemistry experiments: rabbit polyclonal anti-angiostatin, mouse monoclonal anti-endostatin, rabbit polyclonal MMP-2, rabbit polyclonal MT1-MMP and rabbit polyclonal MMP-9 antibodies. These antibodies were purchased from Abcam (Cambridge, MA). Anti-mouse VEGF antibody was purchased from R&D systems (Minneapolis, MN). Purified rat anti-mouse CD 31 antibody was obtained from BD Pharmingen; rabbit anti-connexin 37 antibody from Invitrogen. Following fluorescent secondary antibodies for Immunohistochemistry (IHC) were ordered from Invitrogen (Carlsbad, CA): Texas Red raised in mouse, Alexa Fluor 488, 594 raised in rabbit and Alexafluor 647 raised in rat.

Immunoblot

Western blot was performed on heart tissue homogenates using 10% SDS-PAGE as described previously (Sen *et al.*, 2012). BCA method was used to estimate total protein, and 25 µg of protein was loaded in each well of electrophoresis gels. After electrophoresis, proteins were transferred to polyvinylidenedifluoride membrane (PVDF), blocked with 5% fat-free milk and blotted with respective antibodies. As a loading control, GAPDH was used. The bands were normalized with GAPDH controls.

RNA extraction and quality assessment

Total RNA from mouse heart was isolated using Trizol reagent (Gibco BRL) following manufacturer's instructions. RNA quality was assessed by Nano Drop ND-1000 and only highly pure quality RNA (260/280–2.00 and 260/230–2.0) was used for RT-PCR studies.

RT-PCR

cDNA was prepared using promega kit. The reverse transcription programme was 25 °C for 10 min, 42 °C for 50 min, and then 70 °C for 15 min. RT-PCR was performed for mRNA expression of MMP-2, -9, VEGF, angiostatin, in study groups using ImProm-II™ Reverse Transcription system kit (Promega Corporation, Madison, WI, cat # A3800) as described

earlier (Givvimani *et al.*, 2012). The primer sequences were the same as reported earlier (Givvimani *et al.*, 2012). The RT-PCR product was electrophoresed on 1% agarose gel in TAE with 0.008% ethidium bromide.

Zymography

Gelatin zymography was performed using 0.01% gelatin in gel. Heart tissues were minced into small pieces in ice-cold extraction buffer (1:3 w/v) containing 10 mM/L cacodylic acid, 20 ZnCl₂, 1.5 NaN₃, and 0.01% Triton X-100 (pH 5.0) and incubated overnight at 4 °C with gentle shaking. The homogenate was then centrifuged for 15 min at 1500 g and supernatant was collected. Protein concentration in the sample was measured using Bradford method, and 50 µg of the protein was electrophoretically resolved for each sample in 10% SDS-PAGE containing 0.01% gelatin as MMP substrate. Gels were washed in 2.5% Triton X-100 for 60 min with one change in between to remove SDS, rinsed in water, and incubated for at least 48 h in activation buffer (50 mmol/l Tris-HCl, 5 mmol/l CaCl₂, and 0.02% NaN₃, pH 7.5) at 37 °C in a water bath with gentle shaking. Gels were then transferred to staining solution (acetic acid:methanol:water, 10:50:40) containing 0.5% Coomassie brilliant blue for 1 h at room temperature. MMP activity in the gel was detected in a dark blue background with white bands. Over-staining was avoided by washing the gel several times with distilled water in room temperature.

Echocardiography

Left ventricular functional status was assessed by transthoracic echocardiography as described elsewhere (Givvimani *et al.*, 2011a). Briefly, echo was performed on mice to achieve two dimensional left ventricle images from an apical view using a SONOS 5500 or 2500; Hewlett-Packard, Inc. (Garnerville, NY, USA) and a 12.5 MHz transducer. Tribromo ethanol (TBE) anaesthesia (intra peritoneal dose of 240 mg/kg body weight), was used to minimize the cardio depressing actions produced by other anesthetics (Papaioannou & Fox, 1993). Mice were depilated with hair removal cream (Nair) and placed on a heating pad to maintain body temperature. The functional status of the heart was assessed by LVIDd, LVIDs, LVPWD and %FS. %FS is the most common method to evaluate left ventricular function in murine echocardiography (Rottman *et al.*, 2007).

Pressure-volume study

Left ventricular functional assessment was also performed using Millar P-V loop study as described earlier (Givvimani *et al.*, 2011a) and the percentage ejection fraction (%EF) was reported.

Cryosectioning

After euthanizing the mice, heart tissue was harvested and washed thoroughly in phosphate buffered saline (PBS) and preserved in Peel-A-Way disposable plastic tissue embedding moulds (Polysciences, Inc., Warrington, PA) having tissue freezing media (Triangle Biomedical Sciences, Durham, NC) at 70 °C until further use; 5 µm thickness tissue sections were made using Cryocut (Leica CM 1850, Leica Microsystems Inc., Buffalo Grove, IL, USA) and placed on super frost plus microscope slides, air dried and processed for staining.

Immunohistochemistry

5 µm thick frozen sections of the heart were used to perform immunohistochemistry (IHC) following standard IHC protocol (Abcam) as described previously (Givvimani *et al.*, 2010). Following overnight primary antibody application, secondary antibodies were applied as described in the protocol and stained slides were mounted and visualized with fluorescence

by a laser scanning confocal microscope (Olympus FluoView1000, Pittsburgh, PA, USA) with an appropriate filter.

Masson's trichrome staining

Fibrosis or collagen deposition in tissue sections was assessed by Masson's trichrome (Richard-Allan Scientific, Kalamazoo, MI) staining as per the manufacturer's instructions. Collagen appeared as a blue colour.

Image Proplus software

Images from Immunohistochemistry and Masson's trichrome staining were analysed with Image Proplus software (Image Proplus software; Media Cybernetics, Inc., Rockville, MD, USA). Image analysis was done using Image-Pro software in confocal images taking different optical fields at random into consideration while the densitometry analysis was done using Bio-Rad software (Bio-Rad Software, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Averaged data are expressed as mean $\pm$ SE from $n = 5$. Statistical analysis was performed using commercially available Primer of Biostatistics software (Biostatistics software: Primer of Biostatistics, Seventh edition, Mc Graw Hill Medical). One-way analysis of variance (ANOVA) was performed to test for treatment effects, and differences between groups were determined using Tukey's post-hoc test. Most of the comparisons were between WT AB to WT control and TIMP-2 AB to WT AB groups. A p value < 0.05 was considered to be significant.

Results

Decreased MMP-2 activity was present in TIMP-2^{-/-} aortic banding mice

Genotyping data suggest that TIMP-2^{-/-} mice were homozygous knockout (Figure 1A). As TIMP-2 plays an important role in activating pro MMP-2 to active MMP-2, we did gelatin zymography to see the MMP-2 activity. Our results showed that MMP-2 activity was significantly lower in TIMP-2^{-/-}-aortic banding mice compared to wild type controls (Figure 1B). These results suggest that TIMP-2^{-/-} mice have decreased expression of active MMP-2.

Lack of TIMP-2 exacerbates left ventricular dysfunction during pressure overload

Left ventricular function assessed by the echocardiography (%FS) and pressure volume studies revealed severe left ventricular dysfunction in TIMP-2^{-/-} aortic banding animals compared with wild type aortic banding animals. The percentage ejection fraction (%EF) in TIMP-2^{-/-} aortic banding mice was around 25 as compared to 40 in wild type aortic banding mice. The left ventricular end diastolic dimension (LVEDd) was significantly greater in TIMP-2^{-/-} AB mice (Figure 2). These results suggest that lack of TIMP-2 exaggerates the left ventricular dysfunction during pressure overload condition.

Decreased connexin expression and increased collagen deposition or fibrosis was present in TIMP-2^{-/-} mice with aortic banding

Connexins 37 and 43 are predominantly expressed gap junction proteins in the myocardium. We have reported that disruption in the endothelial-myocyte (E-M) coupling due to decreased gap junction proteins lead to arrhythmia and heart failure. Our results suggest that expression of connexins 37 and 43 was significantly decreased in TIMP-2^{-/-} aortic banding heart compared to wild type controls (Figure 3). Collagen staining assessed by Masson's trichrome staining showed excessive deposition of fibrosis in the TIMP-2^{-/-} mice compared

with wild type mice during pressure overload. Collagen deposition was mostly at perivascular and peripheral regions in wild type aortic banding where as it was more generalized in distribution in TIMP-2^{-/-} aortic banding mice (Figure 4). These results suggest that decreased connexin expression in TIMP-2^{-/-} aortic banding could lead to E-M uncoupling and predisposes to increased fibrosis leading to heart failure.

Extracellular matrix remodelling and disruption of balance between angiogenesis and anti-angiogenesis in TIMP-2^{-/-} aortic banding mice

Pressure overload on heart results in altered expression of matrix metalloproteinases resulting in extracellular remodelling. MMP-2 and MMP-9 are two important gelatinases that induce remodelling in the myocardium. We have reported that expression of pro-angiogenic MMP-2 along with VEGF and CD31 was decreased while expression of MMP-9 and anti-angiogenic factors, angiostatin and endostatin was increased during heart failure. Similarly, results from this study showed decreased angiogenesis in TIMP-2^{-/-} AB mice as suggested by decreased expression of CD31 and VEGF (Figures 5 and 6). Interestingly expression of MMP-2 was also significantly decreased in TIMP-2^{-/-} AB mice compared to their corresponding controls (Figures 5, 8 and 9). Conversely expression of anti-angiogenic factors like angiostatin and endostatin along with MMP-9 was significantly increased in TIMP-2^{-/-} aortic banding mice compared to wild type control mice suggesting exaggerated heart failure (Figures 7, 8 and 9). TIMP-2 also inhibits MT1-MMP (MMP-14) and our results showed increased expression of MMP-14 in TIMP-2^{-/-} AB mice compared the wild type controls (Figure 8). These results suggest that expression of proangiogenic MMP-2, VEGF and CD31 was decreased while that of MMP-9 and endostatin and angiostatin was significantly increased in TIMP-2^{-/-} AB leading to exaggerated heart failure.

Discussion

Hypertension or pressure overload on the heart is a major risk factor of heart failure. After initial compensatory cardiac hypertrophy, sustained overload leads to heart failure. We and others have shown that pro-angiogenic MMP-2 is induced and expressed during hypertrophy. Lack of MMP-2 results in decreased angiogenesis causing heart failure. MMP-2 is both activated and inhibited by TIMP-2. TIMP-2 activates pro-MMP-2 to MMP-2 but when there is excess TIMP-2 then it inhibits MMP-2 (Brew & Nagase, 2010; Butler *et al.*, 1998; Strongin *et al.*, 1995). MT1-MMP forms a tri-molecular complex with MMP-2 and TIMP-2 during activation of pro-MMP. Interestingly TIMP-2 also has an inhibitory action on MT1-MMP.

The rationale of this study was to show that the pressure overload heart failure is much exacerbated or accelerated in TIMP-2 knockout mice compared to wild type mice. We verified the activity of MMP-2 using zymography as TIMP-2 is the main regulator of MMP-2 activity and our results confirmed the decreased MMP-2 activity in TIMP-2^{-/-} aortic banding mice compared to that of wild type aortic banding mice (Figure 1B). As reported previously, by 8 weeks, ascending aortic banding mice attain heart failure stage with decreased left ventricular function. LV function was assessed by echocardiography and pressure volume loop and our results clearly demonstrated that under similar conditions, TIMP-2^{-/-} mice with aortic banding have very poor left ventricular function (%EF = 25) as compared with wild type aortic banding mice (%EF = 40) (Figure 2). Pressure overload on ventricle leads to extracellular matrix remodelling regulated by MMPs and their tissue inhibitors (TIMPs). MMP-2 induces expression of VEGF and thus increases angiogenesis during compensatory hypertrophy. MMP-9 induces anti-angiogenic factors like angiostatin and endostatin thus decreasing blood supply to hypertrophied myocardium leading to collagen deposition and fibrosis during heart failure. Results from our study showed that

accumulation of fibrosis is significantly more in TIMP-2^{-/-} mice with aortic banding compared to wild type aortic banding mice. Interestingly, expression of MMP-2, VEGF and CD 31 (Figures 5, 6, 8 and 9) was significantly decreased while that of MMP-9, angiostatin and endostatin has significantly increased (Figures 7, 8, 9 and 10) in TIMP-2^{-/-} aortic banding mice compared to wild type aortic banding mice correlating with the degree of damage to ventricle. The decreased expression of VEGF and CD31 (capillary endothelial angiogenesis marker) in the pressure overload mice represents decreased neo-vascularization in both WTAB and TIMP-2KO AB mice but more in TIMP-2KO AB mice leading to increased fibrosis (Figure 4).

The structural abnormalities in the hypertensive heart are not only limited to myocyte hypertrophy but also include medial hypertrophy of the intra myocardial coronary arteries as well as collagen deposition leading to cardiac interstitial fibrosis (Braunwald, 1980). Connexins are the gap junction proteins that are widely present in the myocardium. Previously, we have reported that disruption in endothelialmyocyte coupling due to decreased connexin expression leads to heart failure (Givvimani *et al.*, 2011b). Recently, it was shown that reduced connexin 43 expression predisposes to cardiac fibrosis and ventricular arrhythmia (Jansen *et al.*, 2012). Connexins 37 and 43 play an important role in cardiac conduction and were significantly affected during heart failure (Dupont *et al.*, 2001). As such we examined the expression of these connexins and found significantly decreased expression of connexins 37 and 43 in TIMP-2^{-/-}-aortic banding mice compared with wild type aortic banding mice (Figure 3). Interestingly, collagen staining also showed robust deposition of fibrosis in Timp-2^{-/-} aortic banding mice compared with controls (Figure 4).

The beneficial effect of TIMP-2 over expression was reported in rodent models of atherosclerosis (Johnson *et al.*, 2006). It was reported that TIMP-2 deficiency accelerates post myocardial matrix remodelling and TIMP-2 gene delivery ameliorates it (Kandam *et al.*, 2010; Ramani *et al.*, 2011). MT1-MMP was shown to be responsible for post myocardial remodelling in TIMP-2 deficient mice (Ramani *et al.*, 2011). More recently, though it was reported that pressure overload induced left ventricular failure is much exacerbated in TIMP-2 knockout mice compared to wild type mice, the role of MMP-2, angiogenesis and anti-angiogenic factors in this mechanism were unclear (Kandam *et al.*, 2011). It was shown that reduced MMP-2 activity was directly related to cardiac fibrosis in experimental diabetic cardiomyopathy (Van Linthout *et al.*, 2008). Interestingly, it was also reported that the expression of MMP-2 was increased while mitigating the left ventricular dysfunction and collagen accumulation in experimental myocardial infarction in MMP-9^{-/-} mice (Ducharme *et al.*, 2000). Here we demonstrated that pressure overload in TIMP-2^{-/-} mice exacerbated left ventricular dysfunction by decrease in expression of VEGF and increase in angiostatin and endostatin expression. The possible mechanism during this process could be decreased pro-angiogenic MMP-2 activation by TIMP-2. To our knowledge this is the first study to demonstrate the role of decreased MMP-2 activation in TIMP-2KO condition leading to decreased angiogenesis during pressure overload condition on heart, directing us to further insight in pathogenesis of heart failure and possible interventions.

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Figure 1.

A. Genotyping of wild type and TIMP-2^{-/-} mice. Wild type band shown at 250 Kbp and TIMP-2^{-/-} at 280 bp. B. Zymography data showing the activity of MMP-2 in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks mice. Bar graph represents densitometry values in arbitrary units. * $p < 0.05$ was considered significant compared with wt ctrl and wt AB and # $p < 0.05$ compared with wt AB 8 week group.

Figure 2.

Representative M-mode echocardiography images in sham (wt), Wt AB 8 weeks, TIMP-2/- Ctrl and TIMP-2/- AB 8 weeks. Percentage ejection fraction (%EF) was obtained from Pressure-volume loop study. Bar graphs represent % FS, LVEDd (mm) and % EF values. * $p < 0.05$ was considered significant compared with sham and # $p < 0.05$ compared with wt AB 8 week group.

Figure 3.

IHC staining of heart sections with connexins 37 and 43 antibodies in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks. Expression of Cx 37 is seen as green fluorescence intensity and Cx 43 as red fluorescence intensity. Scale bar – 20 μ m. * p <0.05 was considered significant compared with sham and # p <0.05 compared with wt AB 8 week group.

Figure 4.

Masson's trichrome staining of heart sections in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks. Bar graphs represent percentage collagen. * $p < 0.05$ was considered significant compared with sham and # $p < 0.05$ compared with wt AB 8 week group.

Figure 5.
mRNA expressions of MMP-2 and VEGF in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 wks.

Figure 6. Immunohistochemistry staining for CD 31 (angiogenesis marker) in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks. Scale bar – 20 μm. Bar graph depicts the number of CD 31+ve capillaries. * $p < 0.05$ was considered significant compared with sham and # $p < 0.05$ compared with wt AB 8 week group.

Figure 7.
mRNA expressions of MMP-9 and angiostatin in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks.

Figure 8.

Protein expression of MMP-2, MMP-9, MMP-14 and angiostatin by western blot in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks. * $p < 0.05$ was considered significant compared to sham and # $p < 0.05$ compared with wt AB 8 week group.

Figure 9.

Immunostaining of heart sections with MMP-2 and MMP-9 antibodies in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks. Expression of MMP-2 is seen as red fluorescence intensity and MMP-9 as green fluorescence intensity. Scale bar – 50 μ m.

* $p < 0.05$ was considered significant compared with sham and # $p < 0.05$ compared with wt AB 8 week group.

Figure 10.

IHC staining of heart sections with angiostatin and endostatin antibodies in sham (wt), Wt AB 8 weeks, TIMP-2^{-/-} Ctrl and TIMP-2^{-/-} AB 8 weeks. Expression of angiostatin is seen as green fluorescence intensity and endostatin as red fluorescence intensity. Scale bar – 50 μ m. * p <0.05 was considered significant compared with sham and # p <0.05 compared with wt AB 8 week group.