Resveratrol Treatment of Mice with Pressure Overload-Induced Heart Failure Improves Diastolic Function and Cardiac Energy Metabolism

Sung et al: Resveratrol Treatment in Heart Failure

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Abstract

Background—While Resveratrol (Resv) has multiple beneficial cardiovascular effects, whether Resv can be used for the treatment and management of heart failure (HF) remains unclear. In the current study, we determined whether Resv treatment of mice with established HF could lessen the detrimental phenotype associated with pressure overload-induced HF and identified physiological and molecular mechanisms contributing to this.

Methods and Results—C57Bl/6 mice were subjected to either sham or transverse aortic constriction (TAC) surgery to induce HF. Three weeks post-surgery, a cohort of mice with established HF (%EF<45) was administered Resv (~320 mg/kg/d). Despite a lack of improvement in ejection fraction, Resv treatment significantly increased median survival of mice with HF, lessened cardiac fibrosis, reduced gene expression of several disease markers for hypertrophy and extracellular matrix remodeling that were upregulated in HF, promoted beneficial remodeling and improved diastolic function. Resv treatment of mice with established HF also restored the levels of mitochondrial oxidative phosphorylation complexes, restored cardiac AMPK activation and improved myocardial insulin sensitivity to promote glucose metabolism and significantly improved myocardial energetic status. Lastly, non-cardiac symptoms of HF such as peripheral insulin sensitivity, vascular function, and physical activity were improved with Resv treatment.

Conclusions—Resv treatment of mice with established HF lessens the severity of the HF phenotype by lessening cardiac fibrosis, improving molecular and structural remodeling of the heart, and enhancing diastolic function, vascular function and energy metabolism.

Key Words: heart failure, resveratrol, cardiac remodeling, diastolic function
Despite existing therapies for heart failure (HF), the current one-year mortality rate after diagnosis of symptomatic HF remains at 25-40%\textsuperscript{1}. As such, new strategies to treat this debilitating syndrome and improve the quality and length of life of patients with systolic HF must be developed. While recent work has implicated resveratrol (Resv), a naturally occurring polyphenol, as a potential treatment of HF\textsuperscript{2-4}, the vast majority of these studies either use Resv to prevent the development of HF\textsuperscript{2-6} or inappropriately label modest cardiac dysfunction as HF\textsuperscript{3}. As such, despite many claims to the contrary, it has currently not been clearly established if Resv can be used as a treatment for chronic HF.

An exception to the aforementioned previous studies is the work by Kanammori \textit{et al.},\textsuperscript{4} which showed that Resv treatment of mice with myocardial infarct (MI)-induced HF reversed adverse remodeling and improved cardiac function, suggesting that Resv may be an effective treatment for HF. However, the beneficial effects of Resv in this setting were largely attributed to preventing infarct expansion and cell death instead of treating a stable condition. As a result, it remains to be determined whether or not Resv can be used as a treatment for established HF in a model that precludes confounding effects associated with infarct expansion. This is particularly important as the majority of HF patients with symptomatic HF have an established scar\textsuperscript{7} and beneficial therapies must occur independent from preventing infarct expansion.

Based on complications associated with rodent models of MI-induced HF, we employed a transverse aortic constriction (TAC) mouse model of pressure overload-induced HF that does not involve an infarct and scar tissue formation. This model also allowed us to test the effectiveness of Resv treatment in other clinical conditions that are common causes of HF such as pressure-overload resulting from hypertension or aortic valvular stenosis and coronary artery disease\textsuperscript{8}. Using this model we show that acute Resv treatment improves diastolic function,
reduces HF-induced cardiac remodeling and fibrosis, and restores myocardial glucose oxidation rates in mice with HF. These Resv-mediated effects are associated with a dramatic improvement in survival of mice with established HF.

**Methods**

**Experimental animals**

This investigation conforms with the guidelines of the Canadian Council on Animal Care, and the University of Alberta Animal Policy and Welfare Committee. Male C57Bl6 mice (7 weeks of age) were obtained from Charles River Laboratories and maintained on a 12:12 h light-dark cycle (0600:1800 light) with free *ad libitum* access to food and water for a 1-week acclimatization period. At 8 weeks of age, mice were randomly assigned into groups and were subjected to sham (N=13) or transverse aortic constriction (TAC, N=54) surgery to induce pressure overload-induced HF. Three weeks post-surgery, 80% of the TAC mice (N=45) were considered to be in HF with ejection fraction (EF) < 45% and were randomly allocated into two cohorts of mice where the treatment group was administered Resv (4g Resv/kg AIN-93G diet, Dyets Inc., Bethlehem, PA; N=22) in their diet while the control group (N=23) received regular diet without Resv for an additional 2 weeks. For mice, the dosage of Resv was equivalent to ~320 mg Resv/kg/day as described previously⁹. These doses equate to plasma levels of 10-20 μmol/L in rodents¹⁰.

**Transverse aortic constriction surgery**

TAC surgery was performed as described previously¹¹. Sham mice underwent the same open-chest procedure as the TAC mice but without aortic banding.
Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software. Kaplan Meier survival curves were generated and log-rank test performed to compare curves. Comparisons between groups were performed by Kruskal Wallis non-parametric ANOVA and Dunn’s multiple comparison tests. For further description of materials and methods, refer to the supplementary methods section.

Results

Resveratrol treatment increases survival of mice with heart failure in the absence of changes in systolic function or cardiac hypertrophy

Three weeks post-surgery, mice with established HF (EF<45%; Table 1) were administered diet with (~320 mg Resv/kg BW/day) or without Resv (calculated plasma levels equivalent to patients receiving 150 mg/day). While untreated mice with established HF had a median survival of 35 days, Resv treatment increased survival to 78 days (Fig. 1A), showing that Resv treatment significantly improves survival in mice with established LV pressure overload-induced HF. Based on this finding, subsequent experiments were performed at 5 weeks post-TAC when all untreated mice were still alive and could be compared to Resv-treated mice (Supplemental Table 1).

At 5 weeks post-TAC, mice had significantly reduced EF (Fig. 1B) and increased wet lung weight (Table 2) compared to shams. In addition, compared to these sham controls, mice subjected to TAC displayed significant cardiac hypertrophy, including a 2.2-fold increase in LV mass (Fig. 1C) and 1.3-fold increase in diastolic LV posterior and interventricular septal wall thickness (Table 2). Interestingly, Resv treatment did not improve EF or indexes of ventricular...
hypertrophy in mice with HF as compared to untreated mice (Fig. 1B and 1C). However, while hearts from mice subjected to TAC were markedly dilated as shown by significant increases in end-diastolic LV internal volume and diameter (Fig. 1D and 1E), Resv appeared to modestly reduce both LV chamber size and end diastolic and systolic volumes by nearly 15% (Fig. 1D and 1E, Table 2). These effects are particularly important since the severity of LV dilatation and remodeling is a strong predictor of long-term survival\textsuperscript{12,13}.

In order to determine what effect Resv has on gene expression of established molecular markers of pathological cardiac hypertrophy and HF (such as \textit{anf}, \textit{bnp}, \textit{mhc}\textbeta, and \textit{ska}, which are generally induced in hypertrophied rodent hearts)\textsuperscript{14} we measured these in all groups. Cardiac expression of fetal genes including \textit{anf}, \textit{bnp}, \textit{mhc}\textbeta and \textit{ska} all returned to near baseline values of sham mice when mice with established HF were administered Resv (Fig. 1F). This profound reduction in molecular markers of cardiac hypertrophy by Resv indicates that improvements at the transcriptional level may occur acutely in response to Resv and that translation of these molecular changes into structural changes of the heart may require more time to manifest. Alternatively, this may suggest that the regression in markers of pathological hypertrophy is accompanied by a switch to adaptive physiological hypertrophy in order to maintain cardiac output in response to chronic pressure overload.

**Resveratrol treatment improves diastolic function of mice with heart failure**

Although Resv treatment did not have an effect on systolic function, Resv did improve diastolic function in mice with established LV pressure overload-induced HF. Indeed, LA enlargement induced by pressure overload was reduced by 25% with Resv treatment (Fig. 2A). As the LA dilates in response to increasing LV pressure this is a valuable physiological marker of the
duration and severity of diastolic dysfunction\textsuperscript{15}. Furthermore, Resv treatment was found to reduce mitral E/A ratio (Fig. 2B) and E/E' (Fig. 2C) compared to untreated TAC mice further confirming improved diastolic function with Resv treatment. Together, these data suggest that Resv improves atrial function and/or improves the diastolic filling properties of the LV. These findings underscore the importance that diastolic function plays in HF and are in agreement with the concept that the severity and prognosis of HF are more closely correlated to diastolic filling abnormalities rather than EF\textsuperscript{16}.

**Resveratrol restores mitochondrial protein content and promotes glucose oxidation in heart failure**

Studies from both humans\textsuperscript{17,18} and animals\textsuperscript{19,20} suggest that defects in cardiac mitochondrial oxidative phosphorylation and substrate selection contribute to the development and progression of HF. Importantly, we show that protein levels of oxidative phosphorylation (OXPHOS) complexes were reduced in hearts from mice with HF (Fig. 3A and 3B) and Resv treatment restored these levels similar to sham controls (Fig. 3A and 3B). Consistent with previous reports\textsuperscript{2,21} Resv prevented the inactivation of AMP-activated protein kinase (AMPK; Fig. 3C) in hearts from HF mice. Interestingly, activation of Akt was unchanged between groups during fasting when plasma insulin levels are low (Fig. 3D). However, the blunted insulin-induced Akt activation normally observed in HF was improved with Resv treatment (Fig. 3E), suggesting that Resv improves myocardial insulin sensitivity in the failing heart. As restored levels of OXPHOS complexes, AMPK activation and improved insulin sensitivity induced by Resv could contribute to improved myocardial energetic status via the stimulation of glucose metabolism\textsuperscript{10,22}, we measured oxidative metabolism in *ex vivo* perfused working hearts. While both myocardial
glucose oxidation (Fig. 3F) and fatty acid oxidation (Fig. 3G) rates were reduced in the failing heart. Resv treatment increased glucose oxidation levels (Fig. 3F). Together these data suggest that improved myocardial energetic status contributes to the improved diastolic function and increased survival observed in Resv-treated HF mice.

**Resveratrol reduces cardiac fibrosis and markers of cardiac stress in mice with heart failure**

Excessive cardiac fibrosis from chronic pressure overload affects myocardial compliance resulting in increased myocardial stiffness, which is a major determinant of diastolic dysfunction\(^{23, 24}\). Because of this, we investigated the mechanisms responsible for Resv improving diastolic function in mice with established HF by examining cardiac fibrosis and matrix remodeling. Masson’s trichrome staining and immunoblot analysis showed that collagen was dramatically increased in hearts from mice with HF. Importantly, these parameters were reduced with Resv treatment (Fig. 4A-C) demonstrating that Resv reduces the degree of cardiac fibrosis in mice with established HF. In addition, expression of cardiac markers of inflammation such as TNF-\(\alpha\), IL-1\(\beta\) and TGF-\(\beta\) were similar between HF mice treated with or without Resv (data not shown). In agreement with a reduction in collagen deposition and beneficial cardiac remodeling, transcription markers of fibrosis and remodeling were also significantly reduced in Resv-treated HF mice. In fact, numerous genes involved in extracellular matrix and fibrotic remodeling such as \textit{Col 1, Col 3, TIMP-1, TIMP-2, TIMP-3, and TIMP-4}, were almost completely returned to baseline levels observed in hearts from untreated sham control mice (Supplemental Table 2; Fig. 4D and 4E). Furthermore, gene expression of matrix metalloproteinase (MMP)-2, which is known to degrade type I collagen and play an important
role in extracellular matrix remodeling, was significantly increased in hearts from TAC mice (Fig. 4E), which is consistent with studies of pressure overload in humans\textsuperscript{25} and animal models\textsuperscript{26,27}. In agreement with MMP-2 being involved in detrimental remodeling, treatment with Resv reduced MMP-2 levels in hearts from mice with HF (Fig. 4E). These findings suggest that the ability of Resv to lessen cardiac fibrosis and reduce molecular events contributing to LV remodeling may be involved in improved LV diastolic filling properties and reduced LA size in mice with established HF.

**Resveratrol treatment does not alter SIRT1 expression but increases antioxidant defense protein levels in hearts from mice with heart failure**

Resv has been shown to be a potent activator of silent information regulator 1 (SIRT1)\textsuperscript{2} and as such we investigated whether SIRT1 is altered by Resv treatment in HF. Contrary to previous studies\textsuperscript{2}, SIRT1 protein levels were not changed in TAC hearts nor did Resv treatment alter SIRT1 levels (Fig. 5A), suggesting that Resv may not act through the SIRT1 pathway to mediate the beneficial effects of Resv in HF. In addition, oxidative stress in cardiac tissue, as assessed by myocardial 4-hydroxy-2-nonenal levels (HNE), was not changed in HF mice in the presence of Resv compared to untreated HF mice (Fig. 5B). However, cardiac manganese superoxide dismutase (MnSOD) protein expression was significantly reduced in HF compared with control and was restored to control levels by Resv (Fig. 5C). Together, these data suggest that maintaining antioxidant defense enzymes such as MnSOD may be a key factor underlying Resv-mediated protection against oxidative stress in HF.
Resveratrol treatment increases physical activity, insulin sensitivity and improves vascular function in mice with heart failure

Although reduced physical activity is a hallmark feature of HF, studies have shown that low LVEF do not correlate to exercise intolerance in humans with HF and have thus proven to be poor predictors of exercise capacity. Given that diastolic function may be a better determinant of physical inactivity in HF than systolic function, we assessed whether the improvement in diastolic function by Resv treatment had any impact on physical activity. Using the Oxymax lab animal monitoring system with x, y and z-axis activity monitors we show that mice with HF displayed a decline in overall physical activity as measured by reductions in total levels of rearing, grooming and ambulatory activity, and that this was at least partly restored with Resv treatment (Fig. 6A).

Since abnormalities in vascular and endothelial function have been proposed to be important contributors to impaired aerobic capacity and the development and progression of HF, we assessed whether Resv may indirectly increase physical activity via its ability to improve vascular function and ultimately increase oxygen perfusion to the heart and skeletal muscle. Using flow-mediated dilation of the left femoral artery, we show that mice with established HF failed to increase femoral artery flow velocity compared to sham animals following 5 minutes of ischemia (Fig. 6B). In contrast, Resv treatment of mice with HF significantly improved flow-mediated vasodilatation (Fig. 6B). Together, these data show that in the absence of changes in cardiac output, Resv treatment improves vascular function in mice with established HF and this may contribute to the increased physical activity observed in Resv-treated mice with HF by allowing greater peripheral oxygen delivery that may ultimately increase exercise capacity.
Lastly, several lines of evidence point to HF being an insulin resistant state, with the degree of insulin resistance correlating with disease severity and reduced exercise capacity in HF patients\textsuperscript{37,38}. Consistent with this, basal and insulin-induced Akt activation in skeletal muscle were reduced in HF mice (Fig. 6C and 6D). However, Resv treatment restored skeletal muscle insulin sensitivity in mice with HF to levels similar to sham mice (Fig. 6D), which may contribute to increased physical activity in these mice.

**Discussion**

We have recently shown that Resv is cardioprotective in models of hypertension and doxorubicin-induced cardiotoxicity\textsuperscript{36,39}. However, it is not known whether Resv can be used to treat established pressure overload-induced HF and prevent and/or reverse cardiac and vascular remodeling induced by HF. Using the TAC mouse model, we made a number of key findings starting with Resv treatment significantly improving median survival of mice with HF. To investigate the potential physiological and molecular mechanisms that could be responsible for this significant survival benefit, we investigated effects of Resv on cardiac structure and function. Interestingly, while Resv did not improve systolic function it did improve diastolic function in HF mice, as well as modestly reduced LVEDV, LVESV, LV chamber diameter and LA dimensions. Furthermore, Resv treatment reduced cardiac fibrosis and molecular markers of cardiac hypertrophy and remodeling that are commonly observed in the failing heart. These latter findings suggest that Resv may prevent LV remodeling in the setting of HF and ultimately reduce LV stiffness and improve LV diastolic filling properties.

Since cardiac remodeling is an independent contributor to HF progression, the prevention or regression of adverse cardiac remodeling is considered an important therapeutic target in
treatment of this syndrome. Indeed, several large HF studies with pharmacological or device therapy have shown that a reduction in LVESV of at least 10% signifies a clinically relevant reversal of LV remodeling, which is a strong predictor of lower long-term mortality and cardiovascular events\textsuperscript{12,40}. Therefore, the 15% reduction in LVESV in the Resv-treated HF mice in our current study may reflect this degree of positive LV remodeling. Consistent with this, Resv treated mice have a lower mortality rate and reduced HF symptoms (i.e. physical inactivity) compared to untreated HF mice. Interestingly, studies of HF patients have shown that the degree of diastolic dysfunction and not EF influenced survival rate, suggesting that diastolic dysfunction is an important contributor to HF independent of EF\textsuperscript{41,42}. Furthermore, mounting evidence also indicates that LA enlargement is associated with a poorer prognosis and is a powerful predictor of outcomes in patients with HF with reduced EF providing additional prognostic information independent from systolic and diastolic function\textsuperscript{12}. Together, this supports that Resv improves survival rates in mice with established HF in part by improving diastolic function and reducing LA enlargement.

To understand the molecular mechanisms responsible for Resv’s effects in HF, we investigated AMPK which has been shown to be activated by Resv to mediate several beneficial cardiovascular and metabolic effects\textsuperscript{2,21}. We observed increased activation of cardiac AMPK in our Resv-treated HF mice, as well as restored levels of mitochondrial OXPHOS proteins. Consistent with previous reports showing that total myocardial oxidation is diminished in the pressure overloaded heart\textsuperscript{19,43}, we also found that glucose and fatty acid oxidation are reduced in the failing heart. Although this likely contributes to myocardial energetic deficiency in heart failure, it is possible that the failing heart switches to utilizing greater endogenous substrates. However, given the fact that the heart has limited endogenous stores of these substrates\textsuperscript{44,45}, it is
likely that these endogenous stores are not sufficient to meet energetic demand. Resv treatment of mice with HF was associated with increased myocardial insulin sensitivity and rates of glucose oxidation. Since myocardial relaxation is a highly energy consuming process where ATP hydrolysis is necessary for myofilament inactivation and active sequestration of calcium by the sarcoplasmic reticulum\(^{46}\), increased ATP supplied from glucose oxidation may be partially responsible for improved diastolic function in Resv-treated HF mice.

Exercise intolerance is widely recognized as a primary symptom in patients with chronic HF, which limits physical activity and reduces quality of life\(^{28}\). In addition, a growing body of evidence has shown that exercise training benefits the health of HF patients by improving exercise capacity, peak exercise performance, oxygen extraction in the periphery, as well as reducing cardiac events\(^{47}\). Since Resv has been suggested to be an exercise mimetic\(^{22,48}\), we further hypothesized that Resv treatment of mice with HF may also improve physical activity and that this may also contribute to improvement in overall health and subsequent improved survival that we observe in these mice. Consistent with our hypothesis, mice with pressure overload-induced HF displayed reduced overall levels of physical activity and had a shorter life span than sham mice. Importantly, Resv treatment greatly improved both of these parameters. Although the reasons for increased physical activity of HF mice treated with Resv are not completely clear, it is well known that alterations in coronary and peripheral vascular function, in particular endothelial dysfunction, may also be one of the mechanisms underlying exercise intolerance and lack of physical activity in HF\(^{49}\). Consistent with this, we show that mice with HF also possess impaired vascular function. In agreement with our previous findings that Resv has vasodilatory properties and improves vascular function\(^{36}\), we show that Resv improved flow-mediated dilatation of the femoral artery of mice with pressure overload-induced HF. Overall,
we conclude from these data that the ability of Resv to improve vascular function in mice with established HF may partly explain how Resv improves total physical activity by increasing blood/oxygen/metabolic substrate delivery to the heart and muscle. However, as we have only measured daily spontaneous activity, it remains unknown if Resv can improve exercise capacity in these mice. Since studies in healthy mice suggest that Resv acts as an exercise mimetic, Resv may also be able to improve exercise capacity in mice with HF\textsuperscript{39}. An important point is that the treatment, although effective, was only for 2 weeks and it is unclear why the benefits are not sustained and/or what other mechanisms are acting at later timepoints. Although TAC has been widely accepted as an appropriate animal model of HF\textsuperscript{50}, the model has limited relationship to most forms of HF because of the persistent LV pressure overload not typically left untreated in most HF patients.

Although we have likely not identified all of the physiological and molecular mechanisms responsible for the beneficial effects of Resv on survival and physical activity in mice with LV pressure overload-induced HF, we show that Resv reduces cardiac fibrosis, improves diastolic cardiac function, improves myocardial glucose utilization, and improves peripheral vascular function. Together, the data presented herein suggest that Resv has multiple beneficial physiological effects in the setting of HF and that these cumulative changes may contribute to lessening the detrimental phenotype associated with pressure overload-induced HF.
Sources of Funding

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Disclosures

None.

References


39. Dolinsky VW, Rogan KJ, Sung MM, Zordoky BN, Haykowsky MJ, Young ME, Jones LW, Dyck JR. Both aerobic exercise and resveratrol supplementation attenuate


Table 1. Physical characteristics and cardiac morphology and function from mice pre-treatment

<table>
<thead>
<tr>
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<th>Sham</th>
<th>TAC</th>
<th>TAC + Resv</th>
</tr>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>23.50 ± 0.35</td>
<td>23.20 ± 0.41</td>
<td>23.00 ± 1.62</td>
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<td>HR, bpm</td>
<td>412 ± 15</td>
<td>471 ± 19</td>
<td>529 ± 8 *</td>
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<td><strong>Wall Measurements</strong></td>
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<td>Corr. LV mass, mg</td>
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<td>122.47 ± 4.75</td>
<td>120.24 ± 7.93</td>
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<td>LVID-diastole, mm</td>
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<td>LVID-systole, mm</td>
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<td>3.41 ± 0.05</td>
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<td>LA diameter, mm</td>
<td>2.03 ± 0.07</td>
<td>2.34 ± 0.11</td>
<td>2.37 ± 0.14</td>
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<td><strong>Systolic Function</strong></td>
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<tr>
<td>EF, %</td>
<td>64.26 ± 3.50</td>
<td>38.12 ± 1.70</td>
<td>36.24 ± 3.54</td>
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<td>FS, %</td>
<td>35.11 ± 2.68</td>
<td>18.66 ± 0.97</td>
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<td>LVEDV, μl</td>
<td>57.98 ± 4.32</td>
<td>78.25 ± 1.57</td>
<td>72.35 ± 3.04</td>
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<td>LVESV, μl</td>
<td>22.81 ± 3.69</td>
<td>47.92 ± 1.65</td>
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<td>CO, ml/min</td>
<td>16.51 ± 1.10</td>
<td>13.62 ± 0.81</td>
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<td>SV, μl</td>
<td>38.96 ± 3.06</td>
<td>30.51 ± 1.78</td>
<td>25.61 ± 1.78</td>
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<td><strong>Diastolic Function</strong></td>
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<tr>
<td>Mitral E/A ratio</td>
<td>1.80 ± 0.12</td>
<td>3.50 ± 0.51</td>
<td>3.48 ± 0.56</td>
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<td>Mitral E Velocity, mm/s</td>
<td>698.34 ± 42.00</td>
<td>870.67 ± 40.67</td>
<td>757.80 ± 24.81</td>
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<td>Mitral A Velocity, mm/s</td>
<td>396.44 ± 24.89</td>
<td>284.29 ± 50.74</td>
<td>330.35 ± 64.22</td>
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<td>E/E’</td>
<td>24.27 ± 2.60</td>
<td>43.20 ± 2.91</td>
<td>46.63 ± 4.29</td>
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<td>15.06 ± 0.89</td>
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<td>IVCT, ms</td>
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<td>46.06 ± 1.48</td>
<td>54.16 ± 3.27</td>
<td>49.32 ± 2.34</td>
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N=11 shams; N=10-14 TAC and TAC + Resv. * P<0.05 vs. sham by Kruskal Wallis and Dunn’s multiple comparison test.
Abbreviations: HR, heart rate; BW, body weight; Corr. LV mass, corrected left ventricular mass; IVS, interventricular septal wall thickness; LVPW, left ventricular posterior wall thickness; LVID, left ventricular internal diameter; EF, ejection fraction; FS, fractional shortening; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume; CO, cardiac output; SV, stroke volume; IVRT, isovolumic relaxation time; IVCT, isovolumic contraction time; ET, ejection time.
Table 2. Physical characteristics and cardiac morphology and function in mice following 2 weeks of vehicle or resveratrol treatment

<table>
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<th>Sham</th>
<th>TAC</th>
<th>TAC + Resv</th>
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<td><strong>Body weight, g</strong></td>
<td>26.20 ± 0.60</td>
<td>23.96 ± 1.03</td>
<td>25.88 ± 0.76</td>
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<td><strong>Wet lung weight, g</strong></td>
<td>0.15 ± 0.01</td>
<td>0.26 ± 0.03</td>
<td>* 0.27 ± 0.04</td>
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<td><strong>HR, bpm</strong></td>
<td>481 ± 20</td>
<td>473 ± 33</td>
<td>481 ± 22</td>
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<td><strong>24 h Food intake, g</strong></td>
<td>4.26 ± 0.19</td>
<td>3.52 ± 0.18</td>
<td>3.50 ± 0.18</td>
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<td><strong>24 h Food intake/BW</strong></td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.14 ± 0.01</td>
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<tr>
<td><strong>Wall Measurements</strong></td>
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<td><strong>IVS-diastole, mm</strong></td>
<td>0.79 ± 0.02</td>
<td>1.07 ± 0.02</td>
<td>* 1.02 ± 0.04</td>
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<td><strong>IVS-systole, mm</strong></td>
<td>1.20 ± 0.06</td>
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<td><strong>LVPW-diastole, mm</strong></td>
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<td>1.03 ± 0.03</td>
<td>* 0.99 ± 0.04</td>
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<td><strong>LVPW-systole, mm</strong></td>
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<td><strong>Systolic Function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FS, %</strong></td>
<td>31.83 ± 2.03</td>
<td>12.41 ± 1.05</td>
<td>* 11.90 ± 1.20</td>
</tr>
<tr>
<td><strong>LVEDV, μl</strong></td>
<td>65.71 ± 4.79</td>
<td>106.00 ± 3.90</td>
<td>* 90.72 ± 4.44</td>
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<tr>
<td><strong>LVESV, μl</strong></td>
<td>26.73 ± 3.81</td>
<td>78.65 ± 4.26</td>
<td>66.35 ± 5.09</td>
</tr>
<tr>
<td><strong>CO, ml/min</strong></td>
<td>19.43 ± 1.26</td>
<td>13.62 ± 0.81</td>
<td>* 12.23 ± 0.86</td>
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<tr>
<td><strong>SV, μl</strong></td>
<td>41.23 ± 2.20</td>
<td>29.37 ± 1.12</td>
<td>* 25.14 ± 2.28</td>
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<tr>
<td><strong>Diastolic Function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mitral E Velocity, mm/s</strong></td>
<td>746.75 ± 20.20</td>
<td>622.28 ± 56.95</td>
<td>737.25 ± 51.06</td>
</tr>
<tr>
<td><strong>Mitral A Velocity, mm/s</strong></td>
<td>473.97 ± 22.77</td>
<td>324.69 ± 36.05</td>
<td>* 509.81 ± 48.75</td>
</tr>
<tr>
<td><strong>IVRT, ms</strong></td>
<td>17.14 ± 0.51</td>
<td>18.24 ± 0.88</td>
<td>18.25 ± 1.08</td>
</tr>
<tr>
<td><strong>IVCT, ms</strong></td>
<td>6.11 ± 0.46</td>
<td>10.71 ± 0.83</td>
<td>* 10.52 ± 1.05</td>
</tr>
<tr>
<td><strong>ET, ms</strong></td>
<td>43.33 ± 1.91</td>
<td>51.98 ± 1.91</td>
<td>* 49.70 ± 1.66</td>
</tr>
</tbody>
</table>

N=11 shams; N=10-14 TAC and TAC + Resv. * P<0.05 vs. sham, † P<0.05 vs. TAC by Kruskal Wallis and Dunn’s multiple comparison test.

Abbreviations: HR, heart rate; BW, body weight; IVS, interventricular septal wall thickness; LVPW, left ventricular posterior wall thickness; FS, fractional shortening; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume; CO, cardiac output; SV, stroke volume; IVRT, isovolumic relaxation time; IVCT, isovolumic contraction time; ET, ejection time.
Figure Legends

Figure 1. Resveratrol treatment increases survival of mice in heart failure and reduces gene markers of disease. (A) Kaplan-Meier survival curve for untreated (n=7) and Resv-treated (n=7) TAC mice. (B) Ejection fraction (%EF), (C) Left ventricular (LV) mass, (D) LV end diastolic volume (LVEDV), (E) LV internal diameter during diastole (LVIDd; n=11-13) and (F) gene expression of markers of cardiac stress (anf, bnp, mhcβ and ska; n=4-6). * P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001. * P < 0.05 vs sham or # P < 0.05 vs. TAC in (F).

Figure 2. Resveratrol improves diastolic function in mice with heart failure. (A) Left atrial diameter, (B) mitral E/A ratio and (C) E/E’ (n=8-13). * P < 0.05.

Figure 3. Resveratrol treatment restores mitochondrial protein content and cardiac glucose metabolism. (A) Representative image of oxidative phosphorylation proteins in the heart including ATP synthase subunit alpha (C-Vα), Complex III subunit Core 2 (C-III), Complex II subunit 30kDa (C-II-30) and Complex I subunit NDUFB8 (C-I-20). (B) Densitometric analysis of cardiac OXPHOS proteins (n=7-8), (C) phosphorylated AMPK (Thr 172) normalized to total AMPK (n=6), (D) basal Akt phosphorylation and (E) insulin-stimulated Akt phosphorylation in the heart, (F) glucose oxidation rates (n=5-6) and (G) oleate oxidation rates (n=5-6) in hearts from sham, TAC and Resv-treated TAC mice. * P < 0.05 vs Sham; # P < 0.05 vs TAC in (B).

Figure 4. Resveratrol reduces cardiac fibrosis in mice with pressure overload-induced heart failure. (A) Representative images of apical heart sections stained with Masson’s
Trichrome at 40x magnification. Scale bars indicate 132 μm. (B) Quantification of collagen staining in histological images expressed as % area (n=3). (C) Collagen type I (COL1) levels as measured by immunoblot analysis in hearts (n=5). Gene expression of (D) col I and col 3 and (E) matrix metalloproteinase (MMP)-2 and tissue inhibitors of metalloproteinases (TIMP-1, -2, -3 and -4) in ventricular tissue from sham and HF mice (n=4-6). * P < 0.05.

Figure 5. Resveratrol treatment does not increase SIRT 1 levels but increases antioxidant levels. Immunoblot analysis of ventricular tissue from sham, TAC and Resv-treated TAC mice measuring (A) SIRT1 protein levels (n=6), (B) HNE adducts (N=3-4) and (C) MnSOD levels (n=6) that were normalized to tubulin as a loading control. * P < 0.05.

Figure 6. Resveratrol improves total physical activity, vascular function and insulin sensitivity in heart failure. (A) Total physical activity during the awake phase as measured by metabolic cages (n=4-7). (B) Peak femoral blood flow velocity before (pre) and at 1 min (post) following 5 min temporary hindlimb ischemia (n=7-9). Representative tissue Doppler images during vascular occlusion (occl) and 1 min after occlusion release (post) shown above. Phosphorylation status of Akt (Ser 473) in gastrocnemius muscle, collected 10 min after a bolus i.p. dose of (C) saline or (D) dose of insulin (5U/kg), normalized to total tubulin levels (n=4-7). *P<0.05.
Figure 1
Figure 2

(A) Left atrial diameter (mm) for Sham, TAC, and TAC + Resv.

(B) Mitral E/A ratio for Sham, TAC, and TAC + Resv.

(C) E/E' for Sham, TAC, and TAC + Resv.

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Figure 3
Figure 4
Figure 5
Figure 6

A) Graph showing physical activity (beam brakes) for Sham, TAC, and TAC + Resv groups.

B) Graph showing left femoral velocity (mm/sec) for Sham, TAC, and TAC + Resv groups.

C) Basal levels of P-Akt and Tubulin for Sham, TAC, and TAC + Resv groups.

D) Insulin-stimulated levels of P-Akt and Tubulin for Sham, TAC, and TAC + Resv groups.
Resveratrol Treatment of Mice with Pressure Overload-Induced Heart Failure Improves Diastolic Function and Cardiac Energy Metabolism

Miranda M. Sung, Subhash K. Das, Jody Levasseur, Nikole J. Byrne, David Fung, Ty Kim, Grant Masson, Jamie Boisvenue, Carrie-Lynn Soltys, Gavin Y. Oudit and Jason R. B. Dyck

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Materials
Primary antibodies were purchased from Cell Signaling Technology, Santa Cruz Biotechnology, EMD Millipore or Mitosciences; human recombinant insulin (Novolin) was from Novo Nordisk Canada Inc. and trans-resveratrol was purchased from Lalilab (Durham, NC).

Transverse aortic constriction (TAC) surgery
In brief, male 8 week-old mice were anesthetized by an intraperitoneal (i.p.) injection of a cocktail of ketamine (100mg/kg) and xylazine (10mg/kg), intubated, and connected to a mouse ventilator (MiniVent, Harvard Apparatus, Holliston, MA). Following midline sternotomy, a double blunted 27-gauge needle was tied to the aorta between the innominate and left common carotid arteries using 6/0 silk suture. The needle was then removed and the chest and skin were sutured closed using 6/0 silk continuous pattern suture. One week post-surgery, trans-stenotic gradients were measured by pulsed-wave Doppler flow studies to confirm similar pressure overload in all groups of mice with pressure gradients ranging from 50-70 mmHg.

Echocardiography and tissue doppler imaging
Mice were mildly anesthetized using 1.5% isoflurane, and transthoracic echocardiography was performed using a Vevo 770 high resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics) as described previously¹.

Flow mediated dilation after temporary femoral artery ischemia
Mice subjected to either sham or TAC surgery were mildly anesthetized with isoflurane (4% induction and 0.75% maintenance). The femoral artery was visualized with a 40-MHz transducer (RMV-704; VisualSonics, Toronto, Canada) and identified by its characteristic flow pattern. A 0.5 cm vertical incision was made in the left regio femoris, the femoral artery and vein were separated and 3 cm 7/0 nylon (Ethicon) suture was passed around the artery upstream of the site visualized by ultrasound. The site was then closed using 6/0 silk (Ethicon) simple continuous suture and the 7/0 nylon suture tails externalized. Reproducible ischemia and reperfusion was achieved by attaching a bulldog clamp to the exteriorized 7/0 nylon ties and using the weight of the clamp as the constant. Flow arrest was confirmed by abrogation of the Doppler signal. Velocity of flow was monitored at baseline (pre) and after 5 min of ischemia the hindlimb was reperfused by release of the occluder. Reactive hyperemia was monitored by measuring blood flow velocity through the femoral artery at 1 min of reperfusion post-ischemia (post) by Doppler flow measurements.

**Ex vivo heart perfusion**

Hearts were perfused in the working heart mode at 11.5 mm Hg preload and 50 mm Hg afterload with Krebs–Henseleit buffer containing 0.8 mmol/L oleate prebound to 3% delipidated bovine serum albumin (BSA), 5 mmol/L glucose, and 50 μU/mL insulin. At the end of 60 min aerobic perfusion, hearts were snap frozen in liquid N₂ with a Wollenberger clamp and stored at -80°C until analysis. Oleate and glucose were labeled using [9,10-3H]oleate and [U-14C]glucose for the metabolic determination of fatty acid and glucose oxidation, respectively.

**Metabolic analysis in vivo**

Total physical activity was measured using the Comprehensive Lab Animal Monitoring System (CLAMS/Oxymax, Columbus Instruments, Columbus, OH) and was calculated by
adding Z counts (rearing or jumping) to total counts associated with ambulatory movement and stereotypical behavior (grooming and scratching) as described previously\textsuperscript{4}.

**Histology**

Masson's trichrome staining of paraffin-embedded apical heart sections were visualized using a Leica DMLA microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Retiga 1300i FAST 1394 CCD camera (QImaging, Surrey, BC, Canada) as described previously\textsuperscript{5}.

**Gene expression**

Cardiac mRNA expression was determined by real time PCR by using Taqman probes. Total RNA was extracted from flash frozen ventricular tissue by using TRIzol RNA extraction method\textsuperscript{6}. 1µg of RNA was subjected to reverse transcription to synthesize cDNA. Real time PCR was performed by taking 5µl of suitable cDNA dilutions from unknown, standard (brain cDNA) and 8µl Taqman master-mix (includes-primers + Probes) that were then loaded on white 384 Light cycler\textsuperscript{®}480 multi well plates supplied from Roche with 18s rRNA as internal control. Samples are loaded in triplicate and the data was analyzed by Light cycler\textsuperscript{®} 480 machine from Roche. Gene expression of cardiac disease markers (ANF, BNP, MHC-β,α-SKA), fibrosis (COL-I,COL-III,TGF-β), inflammation (IL-1β,TNF-α) and extracellular matrix (ECM) remodeling (TIMP-1,TIMP-2,TIMP-3,TIMP-4, MMP-2) were analyzed (Supplemental Table 2).

**Insulin signaling studies in vivo**

A subset of sham, TAC control and Resv-treated TAC mice were fasted for 6 hours and then given i.p. injections with human recombinant insulin (5U/kg). Mice were sacrificed by decapitation 10 min post-injection, and heart and gastrocnemius muscle was rapidly removed, freeze-clamped in liquid nitrogen and stored at \textminus 80°C until time of analysis.
Immunoblot analysis

Denatured samples of ventricular or gastrocnemius muscle homogenates were subjected to SDS-PAGE and proteins were transferred onto a nylon membrane. Subsequent immunoblotting to determine expression of target proteins was employed. Immunoblots were developed using the Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Waltham, MA). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Densitometric data were corrected against tubulin, actin or respective total protein levels as a loading control.
SUPPLEMENTAL TABLES

Supplemental Table 1. Comparison of echocardiographic data from mice pre- and post-Resveratrol treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAC</td>
<td>TAC + Resv</td>
</tr>
<tr>
<td><strong>Wall Measurements</strong></td>
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<td></td>
</tr>
<tr>
<td>Corr. LV mass, mg</td>
<td>122.5 ± 4.8</td>
<td>120.2 ± 7.9</td>
</tr>
<tr>
<td>IVS–diastole, mm</td>
<td>0.93 ± 0.02</td>
<td>0.94 ± 0.04</td>
</tr>
<tr>
<td>LVPW–diastole, mm</td>
<td>0.90 ± 0.02</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>LVID-diastole, mm</td>
<td>4.19 ± 0.04</td>
<td>4.05 ± 0.07</td>
</tr>
<tr>
<td>LA diameter, mm</td>
<td>2.34 ± 0.11</td>
<td>2.37 ± 0.14</td>
</tr>
<tr>
<td><strong>Systolic Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF, %</td>
<td>38.12 ± 1.70</td>
<td>36.24 ± 3.54</td>
</tr>
<tr>
<td>FS, %</td>
<td>18.66 ± 0.97</td>
<td>17.30 ± 1.94</td>
</tr>
<tr>
<td>LVEDV, µl</td>
<td>78.25 ± 1.57</td>
<td>72.35 ± 3.04</td>
</tr>
<tr>
<td>LVESV, µl</td>
<td>47.92 ± 1.65</td>
<td>45.55 ± 3.96</td>
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<tr>
<td><strong>Diastolic Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitral E/A ratio</td>
<td>3.50 ± 0.51</td>
<td>3.48 ± 0.56</td>
</tr>
<tr>
<td>E/E’</td>
<td>43.20 ± 2.91</td>
<td>46.63 ± 4.29</td>
</tr>
</tbody>
</table>

N=10-14 TAC and TAC + Resv. * P<0.05 vs. TAC pre-treatment; † P<0.05 vs TAC + Resv pre-treatment; ‡ P<0.05 vs. TAC post-treatment by Kruskal Wallis and Dunn’s multiple comparison test.

Abbreviations: IVS, interventricular septal wall thickness; LVPW, left ventricular posterior wall thickness; LVID, left ventricular internal diameter; EF, ejection fraction; FS, fractional shortening; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume.
## Supplemental Table 2. Primers used in real time PCR

<table>
<thead>
<tr>
<th>gene</th>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| ANF      | Forward: 5'-GGA GGA GAA GAT GCC GGT AGA-3'  
Reverse: 5'-GCT TCC TCA GTC TGC TCA CTC A-3'  
Probe: 5'-FAM-TGA GGT CAT GCC CCC GCA GG-TAMRA-3' |
| BNP      | Forward: 5'-CTG CTG GAG CTG ATA AGA GA-3'  
Reverse: 5'-TGC CCA AAG CAG CTT GAG AT-3'  
Probe: 5'-FAM-CTC AAG GCA GCA CCC TCC GGG-TAMRA-3' |
| β- MHC   | Forward: 5'-GTG CCA AGG GCC TGA ATG AG-3'  
Reverse: 5'-GCA AAG GCT CCA GGT CTG A-3'  
Probe: 5'-FAM-ATC TTG TGC TAC CCA GCT CTA A-TAMRA-3' |
| A-SKA    | Forward: 5'-CAG CCG GCG CCT GTT-3'  
Reverse: 5'-CCA CAG GGC TTT G TT TGA AAA-3'  
Probe: 5'-FAM-TGA CCG GT A CAT AGA TTG ACT GTT TT ACC TCA TTT TG-TAMRA -3' |
| IL-1β    | Forward: 5'-AACCTGCTGGTGTGACGTTC-3'  
Reverse: 5'-CAGCACGAGGGTTTTTTTGTTGT-3'  
Probe: 5'-FAM-TTAGACAGCTGCACCCACGGCTCGAGATG-TAMRA-3' |
| TNF-α    | Forward: 5'-ACAAGGCTGCCCGACTAC-3'  
Reverse: 5'-TTTCTCCTGGGTGAGATAGCAAATC-3'  
Probe: 5'-FAM-TGCTCCTCACCCACACCCTGCACG-TAMRA-3' |
| Pro-Collagen-I | Forward: 5'-CTCACCTACACAGCACCCTTGTG-3'  
Reverse: 5'-TGACTGTCTTGGCCCAAGTTTC-3'  
Probe: 5'-FAM-CTGCGAGCCTACCAACC-3' |
| Pro-Collagen-III | Forward: 5'-TGCTCTTTGCGATGACATAATCTG-3'  
Reverse: 5'-ATGGGCTCTGGGTTGGG-3'  
Probe: 5'-FAM-ATGAGGAGCCACTAGACT-TAMRA-3' |
| TGF-β    | Forward: 5'-CCTGCAAGACCATTATGGACATG-3'  
Reverse: 5'-ACAGGATCTGCGCCAACGGAT-3'  
Probe: 5'-FAM-CTGTTGAAACGGAAGCGCATGAA-TAMRA-3' |
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<th>TIMP 1</th>
<th>Forward: 5'-CATGGAAAGCCTCTCTGGATATG-3'</th>
<th>5'-AAGCTGCAGGCACTTGATGT-3'</th>
<th>5'-FAM-CTCATCACGGCCGCCTAAGGAAC-TAMRA-3'</th>
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<tr>
<td>TIMP 2</td>
<td>Forward: 5'-CCAGAAGAAGAGCCTGAACCA-3'</td>
<td>5'-GTCCATCCAGAGCCTCAGTC-3'</td>
<td>5'-FAM-ACTCGCTGTCCCATGACTCCTTGAC-TAMRA-3'</td>
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<tr>
<td>TIMP 3</td>
<td>Forward: 5'-GGCCTCAATTACCCGCTACCA-3'</td>
<td>5'-CTGATAGCCAGGGTACCCAAA-3'</td>
<td>5'-FAM- TGCTACTACTTGCTTGTGACCTCCATAMRA-3'</td>
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<tr>
<td>TIMP 4</td>
<td>Forward: 5'-TGCAGAGGGAGACCTGAA-3'</td>
<td>5'-GGTACATGGCACTGCATAGCA-3'</td>
<td>5'-FAM-CCACCAGAAGCCTGGCTGCTCCAAATC-TAMRA-3'</td>
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<tr>
<td>MMP-2</td>
<td>Forward: 5'-AACTACGATGACCGGAAGTG-3'</td>
<td>5'-TGGCATGGCCGAACCTCA-3'</td>
<td>5'-TCTGTCTGACCCGATGACCTATATCCTCG-3'</td>
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<tr>
<td>18S r RNA</td>
<td>Forward: Mm03928990_g1*</td>
<td>Reverse: Mm03928990_g1*</td>
<td>Probe</td>
</tr>
</tbody>
</table>

**Note:** The sequences provided are for TIMP 1-4 and MMP-2, with the forward, reverse, and probe sequences listed in each case. The 18S r RNA sequence is a reference sequence for normalization.
SUPPLEMENTAL REFERENCES


