Tolerance of isolated rabbit hearts to short ischemic periods is affected by increased LV mass fraction

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Short Title

Ischemic tolerance in rabbit heart with increased LV mass

Summary

Hypertrophied hearts are known for increased risk of arrhythmias and are linked with reduced ischemic tolerance. However, still little is known about state characterized only by increased left
ventricle (LV) mass fraction. Seventeen isolated rabbit hearts with various LV mass were divided into two groups according to LV weight/heart weight ratio (LVW/HW ratio), namely group H and L (with higher and lower LVW/HW ratio, respectively) and underwent three short cycles of global ischemia and reperfusion. The differences in electrogram (heart rate, QRS_{max}, mean number, onset and dominant form of ventricular premature beats) and in biochemical markers of myocardial injury (creatine kinase, lactate dehydrogenase - LDH) and lipid peroxidation (4-hydroxy-2-nonenal – 4-HNE) were studied. As compared to group L, hearts in group H exhibited lower tolerance to ischemia expressed as higher incidence and severity of arrhythmias in the first ischemic period as well as increase of LDH and 4-HNE after the first reperfusion. In the third cycle of ischemia-reperfusion, the preconditioning effect was observed in both electrophysiological parameters and LDH release in group H. Our results showed consistent trends when comparing changes in electrograms and biochemical markers. Moreover, 4-HNE seems to be good potential parameter of moderate membrane alteration following ischemia-reperfusion injury.

Key Words
rabbit isolated heart, LV mass, global ischemia, electrogram, 4-hydroxy-2-nonenal

Introduction

Since the coronary artery disease still represents one of the major causes of morbidity and mortality in many countries, ischemia-reperfusion injury is worth of further research. Using various models, it has been intensely studied from many aspects, largely employing biochemical and electrophysiological methods.

Myocardial ischemia-reperfusion injury is a complex pathophysiological process. Ischemic period is
characterized mainly by decreased ATP level leading to ion imbalance and cell acidosis. In cardiomyocytes, the impairment of the membrane transport mechanisms due to lack of ATP results in a decrease of resting transmembrane potential, action potential (AP), amplitude and duration and eventually in altered conduction velocity (Elharrar and Zipes 1977; Shaw and Rudy 1997). The resulting tissue inhomogeneity may lead to arrhythmias, frequently by re-entry mechanism (Elharrar and Zipes 1977). In reperfusion, the reintroduction of molecular oxygen to the ischemic region leads to excessive generation of reactive oxygen species (ROS), the central mediators of reperfusion injury originating from mitochondria (Raedschelders et al. 2012). Superoxide radical (O$_2^-$) is considered the parent radical from which other radicals originate. The hydroxyl radical (·OH) is particularly harmful because cells do not have antioxidant enzymes for its removal. ·OH attacks all sorts of macromolecules. If polyunsaturated fatty acids in membrane lipids are targeted, formation of secondary peroxidation products such as malondialdehyde, acrolein or 4-hydroxy-2-nonenal (4-HNE) follows. 4-HNE is known for its high reactivity and cytotoxic potential (Poli et al. 2008) and also its participation in development of hypertrophy was described (Calamaras et al. 2012). On the other hand, positive effect of ROS is also known (Kalogeris et al. 2014). In ischemic preconditioning, several cycles of ischemia and reperfusion provoke certain changes in signaling pathways leading to protection of the heart. Preconditioned hearts show lower incidence of arrhythmias and reduced infarct size (Halestrap et al 2007).

A specific situation is observed if a mass of the left ventricle significantly increases. Bigger mass of myocardium requires higher oxygen and nutrient supply and may therefore potentiate tissue vulnerability to ischemia. Moreover, higher number of mitochondria may produce even more ROS and damage during reperfusion may escalate (Muntean et al. 2016). In patients suffering from hypertrophic cardiomyopathy, significantly decreased tolerance to ischemia and intensification of
ischemic heart symptoms was previously reported (Efthimiadis et al. 2014). In ischemia-reperfusion studies, numerous models including isolated heart are used due to possibility to analyze multiple parameters in detail without modulation of neurohumoral inputs (Olejníčková et al. 2015). From experimentally used species, rabbit is appropriate model because of electrophysiological similarity of its heart with human myocardium (Kaese et al. 2013). Predisposition of rabbit heart to spontaneous myocardial hypertrophy was previously reported (Chitty 2015). Therefore, the aim of this study was to assess tolerance of isolated rabbit hearts to repeated ischemic insults with respect to various left ventricle mass fraction. Also biochemical parameters which describe moderate alteration of myocardial membranes related to ischemic insult were studied.

**Materials and Methods**

All experiments were carried out with respect to recommendations of the European Community Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by Local Committee for Animal Treatment at Masaryk University, Faculty of Medicine.

*Isolated heart preparation*

Seventeen adult New Zealand rabbits (both sexes, weight 2.2-3.45 kg) were included to the study. The animals were purchased from certified supplier (Velaz, Czech Republic). Animals were premedicated by diazepam (Apaurin, Krka, Slovenia, 2 mg i.m.) and anaesthesitized by mixture of xylazin (Narkamon, Spofa, Czech Republic, 2 mg/kg i.m.) and ketamine (Calypsol, Gedeon Richter, Hungary, 60 mg/kg, i.m.). Heparin (Heparin Léčiva, Zentiva, Czech Republic, 1000 IU/kg) was administered intravenously to avoid blood clotting. To avoid ischemia during preparation the animals were artificially ventilated (ventilator for small laboratory animals, World Precision Instruments, USA). Bilateral thoracotomy was performed and the hearts were excised and placed into cold Krebs–
Henseleit solution (4 °C). The aorta was cannulated, the hearts were fixed to a modified Langendorff apparatus (Olejníčková et al. 2015) and perfused at constant pressure (70 mmHg) with Krebs-Henseleit solution (NaCl, 118.1 mM; NaHCO₃, 27.1 mM; KCl, 4.8 mM; KH₂PO₄, 1.0 mM; MgSO₄, 1.2 mM; CaCl₂, 1.2 mM; glucose, 10.1 mM) continuously aerated by pneumoxyd (95% O₂ and 5% CO₂). The temperature was maintained at 37°C. The hearts were stabilized during first 30 minutes of perfusion. The experimental protocol consisted of 3 repeated ischemia-reperfusion series: 10 minutes-lasting global ischemia induced by stopping of perfusion was followed by 10 minutes-lasting reperfusion (Fig.1). The flow of coronary effluent was measured as an overflow from the bath in which the heart was placed.

Electrogram recording and analysis

During the whole experiment, three electrograms (EGs) were recorded simultaneously by touch-less method using the orthogonal lead system (Kolářová et al. 2010). The signals were amplified by a set of three biological amplifiers DAM50 (World Precision Instruments, USA) and further simultaneously digitized by 16-bit AD converters at a sampling rate of 2000 Hz using a data acquisition multifunction card PCI-6250 (National Instruments, USA). Analysis of electrogram included heart rate, QRS complex parameters and ventricular arrhythmias assessment. The heart rate (HR) was calculated in each 5th minute of experiment and normalized to the end of stabilization period (100%). QRS complexes in EG recorded from middle area of LV were delineated. Duration and maximal deflection of QRS (QRSd and QRSmax, respectively) were then calculated to evaluate possible effect of LV mass changes on ventricular electrical activity. The ventricular arrhythmias were classified into singles, salvos and ventricular tachycardia (VT) according to the Lambeth conventions (Curtis et al. 2013). The total number of ventricular premature beats (VPBs) in each
minute of the experiment was assessed manually.

**Biochemical analysis**

The samples of coronary effluent were collected at the end of stabilization period and at the end of the first and the third reperfusion (P0, P1, and P3 in Fig.1, respectively). Levels of two cardiac enzymes (creatine kinase - CK, lactate dehydrogenase – LDH) were measured immediately using commercial kits (ErbaLachema, Czech Republic) according to the manufacturer’s instructions. The results were expressed in nkat.min⁻¹.

All chemicals used in biochemical analyses of 4-hydroxy-2-nonenal (4-HNE) were of the p.a. or HPLC grade, purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard of 4-HNE was obtained from Cayman Chemical (Ann Arbor, MI, USA). Determination of 4-HNE was performed according to Kinter (Kinter 1996) with slight modification as described previously (Hlaváčová et al. 2015). Briefly, the 4-HNE concentration in coronary effluent was measured by HPLC with UV detection after derivatization by 2,4-dinitrophenylhydrazine and subsequently calculated according to the standard curve and expressed in nmol.min⁻¹.

**Histological evaluation**

Immediately after the end of isolated heart experiment, sample of tissue from the LV lateral wall was collected, embedded in freezing medium and frozen into liquid nitrogen. Samples were then stored at -20°C until further processing. Sections of 10 um thickness were cut by cryostat, dehydrated and stained. For general morphology, hematoxylin and eosin staining was used. Masson’s trichrome staining was used to assess and visualize the extent of fibrosis in myocardium. Microscope Nikon Eclipse Ti-S/L100 (Nikon, Japan) and NIS elements software (Nikon, Japan) was used to process images and to evaluate the resultant pictures.
Distribution of the animals into groups

The body weight (BW) of animals was assessed before experiment followed by preparation of the heart. At the end of experiment, various anatomical characteristics of hearts were measured: heart weight (HW), left ventricle weight (LVW) and left ventricle wall thickness (LVT). Different visual characteristics were noticed during preparation and course of experiment. Retrospectively, for more detailed analysis of heart morphology following indexes were calculated: the heart weight to body weight (HW/BW) ratio, the LV weight to body weight (LVW/BW) ratio and LV weight to heart weight (LVW/HW) ratio. Based on the abovementioned results animals were distributed into two groups according to the value of LVW/HW ratio which represents the LV fraction in the whole heart mass and was the only index suitable for dividing the animals. To calculate the discrimination threshold value, commonly used receiver operating characteristic analysis (Fawcett 2006) was performed. As a result, the threshold ratio was set to 0.57 so the animals with ratio below or equal 0.57 belong to group L (N = 8, 2 females and 6 males) and above 0.57 belong to group H (N = 9, 2 females and 7 males).

Statistical analysis

The statistical analyses were performed using GraphPad Prism® 5 (version 5.01, GraphPad Software, Inc., San Diego, CA, USA) and Matlab R2014b (The MathWorks, Inc., USA). Normality of data distribution was verified using Shapiro Wilk test. Non-parametric Mann-Whitney U test was used to test the differences between L and H groups in following data sets: 1) basic characteristics (body weight, heart weight and others), 2) QRS complex parameters (QRS$_d$ and QRS$_{max}$) in stabilization period 3) number of VPBs in each ischemic period, 4) values of biochemical parameters calculated at the end of stabilization, the first and the third reperfusion periods (see Fig.1). Additionally, the
differences in values of biochemical parameters between various periods of experiment were verified using non-parametric Friedman repeated measurement test followed by multiple comparison Tuky-Kramer test. Correlation analysis (Spearman's rank correlation coefficient $\rho$ calculated for L and H group separately) was performed to examine the relationship between values of different biochemical parameters in particular experimental period and also between values of each parameter calculated in different periods. The values of heart rate were expressed as mean $\pm$ SEM. Differences in the HR between L and H groups were analyzed by parametric Student’s unpaired t-test. The differences between consecutive experimental periods were analyzed by paired t-test. Differences between individual periods of each experiment and its stabilization period were analyzed by one-sample t-test. For all tests, $p < 0.05$ was considered significant.

**Results**

*Basic characteristics of data sets*

No significant differences in anatomical parameters were found between L and H group. LVW/HW ratio was the only parameter in which these groups differed significantly ($p = 0.002$, Mann-Whitney U test). Spearman's $\rho$ between all pairs of basic characteristics were also calculated. Significant correlation ($p < 0.05$) was confirmed between body weight and heart weight ($\rho = 0.86$ in H group), heart weight and left ventricle weight ($\rho = 0.97$ and $\rho = 0.84$ in L and H group, respectively).

*Electrophysiological assessment of ischemic tolerance*

As expected, HR was affected by ischemic insult. In H and L groups, HR decreased in ischemic periods and was almost restored during the reperfusion periods. In group L, the decrease was significant in all ischemic periods with better recovery after the first ischemia when compared with group H. On the other hand, the HR decrease in group H was lower in all ischemic periods with better
recovery in the second and the third reperfusion periods. Almost all HR changes were significant when comparing two consecutive periods of experiment in both groups. However, no significant changes between the L and H groups were found when comparing the same period of experiment (see Fig. 2).

The change in LV anatomy was reflected in value of QRS\textsubscript{max} only. QRS\textsubscript{max} calculated in H group reached significantly higher value (p < 0.05) than that in L group (mean ± SEM): 2.54 ± 1.37 mV and 4.46 ± 1.52 mV in L and H group, respectively.

Last, arrhythmias were evaluated to assess tolerance of hearts to ischemia-reperfusion injury. During the first ischemia, the mean number of VPBs was higher in group H (32.9 ± 17.9 vs. 14.5 ± 11.0 in L, respectively). The difference has been found in the onset of VPBs (the 6\textsuperscript{th} minute vs. the 5\textsuperscript{th} minute in H and L, respectively). Salvos dominated in H and singles in L group. During the second ischemia, decrease of the mean number of VPBs was observed in both groups (to 96% and 15% in H and L, respectively). The onset of arrhythmias was delayed (to the 7\textsuperscript{th} minute in both groups). Although the dominant form of arrhythmias during the second ischemia remained unchanged in both groups, there was a worsening in the severity of arrhythmias during the third ischemia (VT in group H and salvos in group L, respectively). In group H, the decrease of total VPBs and also delay of onset of arrhythmias continued (to 14 % and the 9\textsuperscript{th} minute). In group L, the onset of arrhythmias remained the same (the 7\textsuperscript{th} minute), however the total VPBs slightly increased (see Table 1 and Fig.3). None arrhythmias were observed in any reperfusion period in both groups.

Biochemical assessment of ischemic tolerance

The values of biochemical parameters (CK, LDH and 4-HNE) related to flow of coronary effluent are shown in Figure 4. There was no significant difference between group L and H in any determined parameter and phase of experiment. Nevertheless in group H slightly increasing trend in the level of
LDH and 4-HNE after the first ischemic period was found. The trend was more visible in values of 4-HNE, where the difference between stabilization period and the first reperfusion was close to significant \( p = 0.055 \). But in the third reperfusion period the trend in levels of LDH and 4-HNE was not the same: while the level of LDH started to decrease slightly, values of 4-HNE showed continuing increase. The increasing trend of 4-HNE and LDH after the first ischemia was not visible in group L. On the contrary, values of biochemical parameters in the first reperfusion were the same (LDH) or even lower (4-HNE) with slight increase of both parameters in the third reperfusion period. The values of CK don’t show any trend in any group due to wide dispersion of values.

Correlation analysis was performed among values of studied biochemical parameters in particular experimental periods between group L and H and no correlations were found. We also examined the relationship between values of each parameter calculated in different periods in the same group. The correlations were calculated separately for group H and L. Relevant correlations were found only in group H. Positive correlation between values of 4-HNE and LDH in samples P0 and P1 were observed. Moreover, from all followed biochemical parameters, only values of 4-HNE positively correlate among all experimental periods (see Table 2).

**Histological evaluation**

Histological analysis revealed that the cardiomyocytes from H vs. L group did not differ as the short axis dimension is concerned. Representative pictures of midventricular sections stained with Masson’s trichrome are shown in Fig. 5a. In the content of collagen, apparent although insignificant difference between group H and L was found (%blue 1.09 ± 0.69% vs. 0.62 ± 0.25%, for group H and L, respectively, see Fig.5b).

**Discussion**
Cardiovascular diseases are still a leading cause of morbidity and mortality. It is well known that pathologically hypertrophic hearts are at high risk of arrhythmias and sudden cardiac death and may develop into heart failure (Verdecchia et al. 2001, Lorell et al. 2000). Besides other consequences hypertrophy is characterized by increased oxidative stress via production of ROS, which are involved both in the progress of cardiac hypertrophy and also alter directly the function of ion channels (Giordano 2005). Therefore, cardiac hypertrophy is widely studied in experimental and clinical medicine. However, it is still little known about state which precedes manifested hypertrophy. The aim of this study was to find whether even slight increase of LV mass expressed as LVW/HW ratio may change myocardial tolerance to ischemia. Biochemical markers suitable for detection of moderate alteration of membranes during ischemia-reperfusion injury were searched for.

In this work we first focused on characterization of hearts with slight increase of LV mass, which might be therefore at higher risk of later progress in hypertrophy. After more detailed statistical analysis the animals were divided according to LVW/HW ratio into group L (lower values of ratio) and H (higher values of ratio). The assessment of LV enlargement in humans is generally based on calculation of LVW using LV volume parameters estimated noninvasively by echocardiographic or magnetic resonance techniques. Because of large differences in body size of subjects and its close relation to LVW, various formulas have been proposed for LVW normalization by parameters representing the body size, such as body weight (BW), body surface area or body mass index (Foppa et al. 2005). Wolk and co-workers reported a rabbit model of induced hypertrophy where the increase of LVW/BW ratio in group with induced hypertrophy was about 25% comparing to control group (Wolk et al. 2001). In the present study, in group H (animals with spontaneously increased LV mass fraction) the increase is only 4%. So it is evident that elevation of this parameter is not as significant as in hypertrophied hearts. It is in agreement with the results of statistical analysis, in which the
LVW/HW ratio is the only index affected by increase of the LV mass fraction. Our distribution to the group with higher (group H) and lower (group L) LV mass fraction is also supported by a trend toward increased content of collagen in myocardium and significant increase of maximal amplitude of QRS under non-ischemic condition. Correlation between quantity of collagen and sudden cardiac death in patients with LV hypertrophy was shown in study of Tamarappoo and co-workers (Tamarappoo et al. 2012). Increased $\text{QRS}_{\text{max}}$ reflects increased LV mass; the Framingham study reported that only the increased voltage of QRS complex is independent risk of cardiovascular mortality (Kannel 1970). These findings support the distribution according to the left ventricle weight to heart weight ratio, which more sensitively demarks hearts with bigger left ventricle mass and changes connected with this condition.

After distribution of animals into group L and H the tolerance of hearts in both groups to ischemia-reperfusion injury was examined. In group H, hearts were more susceptible to ischemic insults expressed both by higher mean number of arrhythmias and their higher severity in both the first and the second ischemic period. Different redox signaling in the hearts with increased LV mass (Sag et al. 2014) may explain their higher susceptibility to ischemia and reperfusion. This is consistent with other studies, where the relation between left ventricular hypertrophy and higher incidence of arrhythmias was manifested (Chatterjee et al. 2014). In the third ischemic period, reduced total VPBs number and the delayed onset was observed in group H. This effect may result from ischemic preconditioning. It is supported by better recovery of HR after the second ischemia and lower decrease of heart rate in the third ischemic period in this group.

For biochemical analyses, three parameters were chosen. Creatine kinase and lactate dehydrogenase (LDH) are rough parameters of direct myocardial injury and cell necrosis. The third parameter - 4-hydroxynonenal (4-HNE) - is a marker of lipid peroxidation which occurs mainly in reperfusion
(Eaton et. al. 1999). From abovementioned parameters, only the values of LDH and 4-HNE showed some differences. In group H, the increase of LDH and 4-HNE after the first reperfusion, in case of 4-HNE nearly significant, confirms higher susceptibility of these hearts to the ischemic insult comparing with group L. This finding is supported by study of Blasig and co-workers where significantly higher release of 4-HNE in group of hypertrophic hearts compared with control group after global ischemia was proven (Blasig et al. 1995). It is also known that cardiac hypertrophy is characterized by increased glucose uptake with reduction in phosphocreatine/ATP ratio described even in patients without developed hypertrophy (Crilley et al 2003). It is possible that this situation of bioenergetic deficit is a stimulus, which causes higher susceptibility of hearts in group H to another energetically complicated condition, which ischemia undoubtedly is. In the third reperfusion, the level of LDH restores more or less to the stabilization level, but the amount of 4-HNE keeps slight increase and shows wider variability as demonstrated in correlation analysis. It might indicate that preconditioning affected LDH release, while moderate and fast non-enzymatic changes of lipoperoxidation on membrane continue with further ischemic periods despite the preconditioning. Such a rough parameter as LDH does not reflect these changes. In addition, there are some associations among electrophysiological and biochemical changes, as described in our previous work discussing correlations between ischemic-induced arrhythmias and production of hydroxyl radicals, which are known precursors of lipid peroxidation (Paulová et al. 2013). For comparison, the animals were divided according to their body weight as a rough anatomical parameter (below and above 3kg). Results revealed that the differences in electrophysiological as well as biochemical results were minimized and less homogenous than when groups H and L were compared, even when the animals differed significantly not only in body weight but also in LV weight, which is used in assessment of LV enlargement.
In summary, certain trends in incidence and severity of arrhythmias and values of biochemical markers were found between the groups with lower and higher left ventricle mass expressed by LVW/HW ratio, which attenuate after dividing the animals into groups according to body weight. Hearts with higher LV mass fraction showed lower tolerance to ischemic insult. From biochemical markers studied, 4-hydroxynonenal is a promising marker of moderate membrane alteration following ischemia-reperfusion injury. Outcomes of this study may be beneficial for future research in the field of the first pathological steps of developing myocardial hypertrophy.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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References

BLASIG IE, GRUNE T, SCHÖNHEIT K, ROHDE E, JAKSTADT M, HASELOFF RF, SIEMS WG: 4-Hydroxynonenal, a novel indicator of lipid peroxidation for reperfusion injury of the


| Table 1. Mean number of different types of VPBs in group L and H. |
|---|---|---|---|---|
| Period | Group | Singles | Salvos | VT |
| | | | | | Total |
| I1 | L | 11.1 ± 11.1 | 3.1 ± 2.6 | 0.3 ± 0.3 | 14.5 ± 11.0 |
| | H | 6.8 ± 3.7 | 22.2 ± 14.3 | 3.9 ± 3.3 | 32.9 ± 17.9 |
| I2 | L | 2.2 ± 1.5 | 0 | 0 | 2.2 ± 1.5 |
| | H | 3.3 ± 2.3 | 23.8 ± 14.8 | 4.7 ± 0.9 | 31.8 ± 18.1 |
| I3 | L | 2.6 ± 2.4 | 4.2 ± 3.8 | 0 | 6.8 ± 5.9 |
| | H | 0.2 ± 0.2 | 1.0 ± 1.0 | 3.3 ± 3.3 | 4.5 ± 3.4 |

Values are means ± SEM; I1, I2, I3 – ischemia; VT – ventricular tachycardia.

| Table 2. Correlations between the values of different biochemical parameters within experimental period and correlations between the values of biochemical parameter from various experimental |
periods in group H

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Period</th>
<th>Spearman's ρ</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH vs. 4-HNE</td>
<td>P0</td>
<td>0.862</td>
<td>0.009*</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>0.700</td>
<td>0.043*</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>0.261</td>
<td>0.594</td>
</tr>
</tbody>
</table>

|                        | P0 vs. P1 | 0.850        | 0.012*  |
| 4-HNE                 | P1 vs. P3 | 0.841        | 0.044*  |
|                        | P0 vs. P3 | 0.638        | 0.200   |

P0, P1, P3 – samples of coronary effluent taken in the end of stabilization period, the first and the third reperfusion. *p<0.05

**Legends to the Figures**

Figure 1. Design of experimental protocol; I - ischemia, R - reperfusion; P 0, 1, 3 - collection of coronary effluent samples at stabilization, the first and the third reperfusion, respectively.

Figure 2. Mean values of normalized HR for L and H group. HR values significantly different from those in previous experimental phase are depicted with *, ** and *** for p<0.05, p<0.001 and p<0.0008, respectively. I - ischemia, R – reperfusion
Figure 3. Distribution of VPBs in each minute over the first, the second and the third ischemia (top, middle and bottom panel, respectively) in group L and H.

Figure 4. Values of biochemical parameters CK (a), LDH (b) and 4-HNE (c) for L and H group in different periods of experiment. Displayed as median, the edges of the box indicate 25th-75th percentiles. P0, P1 and P3 – samples of coronary effluent at the end of stabilization, the first and the third reperfusion, respectively.
Figure 5. Histological analysis of heart tissue;

5a Representative pictures of midventricular section of the left ventricle in group H (H, left) and group L (L, right). Collagen is stained blue (Masson’s trichrome staining). A bar represents 100 µm

5b Ratio of collagen (stained blue) to non-collagen tissue (stained red) in the Masson trichrome staining (in %). Displayed as median, the edges of the box indicate 25th-75th percentiles