Connexin43 Expression in Human Hypertrophied Heart Due to Pressure and Volume Overload

Short title: Connexin43 in left ventricular hypertrophy in humans

[1,5]Christian Vetter, MD, [2,5]Martin Zweifel, MD PhD, [1]Christian Zuppinger, PhD, [1]Thierry Carrel, MD, [3]David Martin, PhD, [3]Jacques-Antoine Haefliger, PhD, [1,4]Etienne Delacrétaz, MD

[1]Swiss Cardiovascular Center Bern, University Hospital, Bern, Switzerland

[2]Division of Medical Oncology, University Hospital, Zurich, Switzerland

[3]Department of Internal Medicine, University Hospital, Lausanne, Switzerland

[4]To whom correspondence should be sent

[5]These authors contributed equally to this work

Corresponding author: Etienne Delacrétaz
Swiss Cardiovascular Center Bern
University Hospital
CH-3010 Bern
Switzerland
Phone: + 41 31 632 21 11
Fax: +41 31 632 42 99
E-mail: etienne.delacretaz@insel.ch

Summary

Background: Left ventricular hypertrophy (LVH) is due to pressure overload or mechanical stretch and is thought to be associated with remodeling of gap-junctions. We investigated whether the expression of Connexin43 (Cx43) is altered in humans in response to different degrees of LVH.

Methods and Results: The expression of Cx43 was analysed by quantitative polymerase chain reaction, western blot analysis and immunohistochemistry on left ventricular biopsies from patients undergoing aortic or mitral valve replacement. Three groups were analysed: patients with aortic stenosis with severe LVH (n=9) versus only mild LVH (n=7), and patients with LVH caused by mitral regurgitation (n=5). Cx43 mRNA expression and protein expression were similar in the 3 groups studied. Furthermore, immunohistochemistry revealed no change in Cx43 distribution.

Conclusion: In comparison with mild LVH or with LVH due to volume overload, severe LVH due to chronic pressure overload is not accompanied by detectable changes of Cx43 expression or spatial distribution.

Key words

Gap junction – Connexin43 – cardiac hypertrophy – aortic stenosis - remodeling

Introduction

Mechanical stress during left ventricular pressure overload is one of the most potent stimuli leading to left ventricular hypertrophy (LVH) and is associated with electro-mechanical remodeling (De Mello 1999; Dupont *et al.* 2001; Peters *et al.* 1997; Peters and Wit 2000; Sepp *et al.* 1996; Teunissen *et al.* 2004). Cardiac gap junction remodeling was demonstrated in response to mechanical stress *in vitro* (Saffitz *et al.* 1999). However, most data are derived from animal experiments. There are conflicting data regarding connexin regulation in relation to cellular hypertrophy *in vivo*. In cardiomyocytes of neonatal mice, acute hypertrophic response elicited by VEGF or stretch increases Connexin43 (Cx43) expression, leading to an increased velocity of propagation of the depolarising wave front (Darrow *et al.* 1996; Zhuang *et al.* 2000). In rat models of chronic LVH however, Cx43 expression was not increased (Haefliger *et al.* 1997b; Haefliger *et al.* 1999). In humans, Cx43 expression is reduced in ventricular myocardium from hypertrophied and ischemic hearts (Peters *et al.* 1993; Teunissen *et al.* 2004). However, this was shown to be most likely due to ischemia (Peters 1996). Thus, little is known on the regulation of the cell-to-cell communication in response to LVH.

The aim of our study was to prospectively investigate the expression of Cx43 in left ventricular biopsies from patients with chronic left ventricular pressure overload with severe LVH versus mild LVH due to moderate to severe aortic stenosis, compared to patients with LVH due to left ventricular volume overload related to severe mitral vave regurgitation.

Methods

Patients selection and human left ventricular myocardial tissue

Patients undergoing aortic valve replacement for severe aortic stenosis without heart failure and without ischemic heart disease were enrolled in the study. They were assigned to two groups according to the degree of LVH. Structural cardiac data were obtained using a commercially available ultrasound system (Acuson 128/XP10c, Acuson, Mountain View, CA, USA) with a 3.5 MHz transducer frequency for M-mode and 2.5 MHz for Doppler recordings. M-mode tracings were quantified according to the recommendations of the American Society of Echocardiography. Left ventricular mass was calculated using the cube formula and overestimation was corrected using the equation proposed by Devereux *et al.* (Devereux *et al.* 1986). Patients with mitral regurgitation undergoing mitral valve replacement were enrolled as subjects with LVH due to volume overload. At the time of cardiac surgery, two biopsies from every patient were taken from the endocardial aspect of the septum of the left ventricle and immediately snap frozen in liquid nitrogen for subsequent analysis. Informed consent was obtained from all patients before inclusion in the study. The protocol used for the experiments complies with the Declaration of Helsinki and was approved by the Human Ethical Committee of the University of Bern.

Western blot analysis and quantification of connexin 43 protein

Frozen left ventricular tissue was powdered and solubilized in a buffer containing 5% SDS supplemented with 5mM EDTA. The DC protein assay reagent kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the protein content. Aliquots of human heart total protein were heated at 95°C for 5 minutes in loading buffer, fractionated by electrophoresis in a 12.5% polyacrylamide gel and

immunoblotted onto Immobilon PVDF membranes (Millipore, Billerica, MA, USA) overnight at a constant voltage of 20 V. Membranes were incubated for one hour at room temperature in PBS containing 5% dry milk and 0.1% Tween 20 (blocking buffer) and then incubated overnight at 4°C with a polyclonal rabbit antibody directed against the C-terminus of Cx43 (Millipore, Billerica, MA, USA) in blocking buffer (final concentration: 5 μg/ml). The membrane was then incubated for 45 minutes at room temperature with horseradish peroxidase (HRP)-coupled goat anti-rabbit immunoglobulins (DAKO, Glostrup, Danmark) in a dilution of 1:2000 and the bands visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Socochim, Rockford, IL, USA). For the detection of β-tubulin, the membrane was stripped using 3.25 ml 1M Tris-Cl pH 6.7, 10 ml SDS 10%, and 350 ul β-mercaptoethanol in 50 ml H_2O and immunoblotting performed using a polyclonal rabbit anti-human β-tubulin antibody (Sigma-Aldrich, Inc., St. Louis, MO, USA). For the quantification of immunoblot bands, pixel densities of scanned immunoblot membranes were analysed using the TinyQuant© software (Norman Iscove, University Health Network, The Ontario Cancer Institute, Toronto, Canada).

Immunofluorescence staining for the detection of connexin 43 protein on histologic sections

For indirect immunofluorescence labelling, 10-µm cryosections were cut in parallel to the fiber's longitudinal axis. Sections were incubated for 30 min. in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA). Sections were then incubated for 12 hours at 4°C with a polyclonal rabbit antibody directed against the C-terminus of Cx43 (Millipore, Billerica, MA, USA) in PBS (final concentration: 5 μg/ml). As secondary antibody, a FITC-labelled goat anti-rabbit IgG antibody was

used (Molecular Probes, Eugene, OR, USA; diluted 1:500). 3 sections per patient, and 10 high power fields per section were visualized, and the distribution pattern morphologically analysed with regard to the localized expression of Cx43 at the poles, and the borders of the myocardial cells, respectively.

Real time reverse transcription-polymerase chain reaction (RT-PCR) for the quantification of connexin 43 mRNA

Quantitative RT-PCR was performed as previously described (Zweifel *et al.* 2002). Frozen left ventricular tissue was homogenized in a 4M guanidine hydrothiocyanate buffer containing 25 mM sodium citrate and 100 mM β-mercaptoethanol. Total RNA was extracted by the acid guanidium isothiocyanate method and yields were evaluated by absorbance at 260 nm. Since Cx43 pre-mRNA does not contain any introns, a DNA digestion step was performed before reverse transcription, in order to remove any contaminant genomic DNA which would also be amplified by the primers and probe during PCR. Quantitative real time RT-PCR was performed for human Cx43 mRNA, eukaryotic 18S rRNA, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Primers and the fluorescent FAM-labeled probe for PCR of the reverse transcribed Cx43 mRNA were designed in-house from published sequences using the PrimerExpress software (Applied Biosystems, Foster City, CA, USA). The sequences of the primers and probe for Cx43 (GenBank accession number AF151980, National Center for Biotechnology Information, National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894, USA, www.ncbi.nlm.nih.gov/Genbank/) were 5‘-GCCCACATCAGGTGGACTGT-3‘ (sense), 5‘-AAGGACACCACCAGCATGAAG-3‘ (antisense), 5‘-CTCGCCCCACGGAGAAAACCATC-3‘ (fluorescence labelled probe). Primers and

probes for the reverse transcribed 18S rRNA and GAPDH mRNA were purchased as predeveloped assay reagent (PDAR) from Applied Biosystems. Real time PCR was performed using the TaqMan Universal PCR Master Mix with 80 ng of reverse transcribed input RNA, and a concentration of primers and probes of 900 nM and 200 nM, respectively, in a final reaction volume of 25 μl in a ABI PRISM 7700 Sequence Detector (Applied Biosystems) according to the manufacturer's protocol. PCR amplification was performed for 40 cycles. PCR amplifications of the constitutively expressed 18S and GAPDH mRNA were performed after reverse transcription as a measure of input RNA. Relative quantity of Cx43 mRNA was then expressed as ratio of the Cx43 mRNA quantity to the quantity of the housekeeping genes 18S, and GAPDH, respectively.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Kruskal Wallis and Mann Whitney-U tests were applied for the analysis of variation among the groups, and to assess significance (p) of the difference between two groups, respectively. $p < 0.05$ was considered significant.

Results

Sixteen patients undergoing aortic valve replacement for severe aortic stenosis were enrolled in the study. They were assigned to two groups according to the degree of LVH, nine with severe LVH, and seven with mild LVH. Five additional patients with mitral regurgitation undergoing mitral valve replacement were enrolled as subjects with LVH due to volume overload. None of the patients had had previous ventricular arrhythmic disorder, and none of them had ischemic heart disease. All patients had a

normal systolic left ventricular function. The clinical characteristics of the patients are summarised in Table 1. Statistical analysis for age, QRS duration, and left ventricular ejection fraction do not show any significant differences between the three groups. The mean aortic valve gradients are similar in both groups with aortic stenosis. The mean left ventricular mass is higher in the group with aortic stenosis and severe LVH, compared to the group with aortic stenosis and mild LVH, and the group with mitral valve disease, respectively. Western blot (Figure 1a) and band density analysis (Figure 1b) demonstrate no significant differences in Cx 43 expression in left ventricular tissue between all three groups.

Representative images of left ventricular sections from the three groups, immunostained with a specific antibody directed against Cx43, are shown in Figure 2: Cx43 is present in all groups and is confined to the cell poles, whereas only little expression is found at the lateral cell border. The distribution pattern of Cx43 is similar in all three groups, as assessed by morphologic microscopic evaluation.

Real time RT-PCR for the quantification of Cx43 in relation to the constitutively expressed house keeping genes 18S, and GAPDH, respectively, does not show any significant difference between the three groups (Figure 3).

Discussion

In this study, large subendocardial left ventricle biopsies taken at the time of aortic or mitral valve replacement allowed us to prospectively compare Cx43 expression in response to different causes and degrees of LVH, at the protein level by western blot, and immunohistochemistry, and at the mRNA level by quantitative RT-PCR.

Patients with chronic LV pressure overload due to aortic stenosis with severe or mild LVH, respectively, as well as patients with volume overload due to mitral regurgitation

were compared. None of the patients had concomitant coronary heart disease or impairment of left ventricular function. Expression levels of Cx43 protein and Cx43 mRNA were identical in patients with LVH due to chronic pressure overload and in patients with LVH due to chronic volume overload. Moreover, the expression of Cx43 was similar in patients with severe LVH compared to patients with mild LVH, and in patients with chronic LV pressure overload versus chronic LV volume overload. Finally, there was no change in Cx43 distribution. Thus, in our study in humans, severe LVH as compared to mild LVH is not associated with a relevant remodeling of Cx43 mediated cell-to-cell communication.

Conflicting data on Cx43 expression have previously been reported in animals with ventricular structural abnormalities (Formigli *et al.* 2003; Haefliger *et al.* 1997b; Haefliger *et al.* 1997a; Uzzaman *et al.* 2000). Increased synthesis of Cx43 and increased conduction velocity have been demonstrated *in vitro* in hypertrophic response to stretch and to factors stimulating cellular growth in neonatal ventricular cardiomyocytes (Darrow *et al.* 1996; Zhuang *et al.* 2000). Cx43 expression is also up-regulated in the early phase of hypertrophy in guinea pigs with renovascular hypertension. In chronic models, no significant changes in Cx43 expression were detected in hypertrophied hearts of rats with hypertension provoked by renal artery clipping or deoxycorticosterone/salt administration, or during inhibition of nitric oxide synthase (Haefliger *et al.* 1997a; Haefliger *et al.* 1997b). However, 8 to 12 weeks after aortic banding, Cx43 was frequently displaced from its usual location at the intercaled disks to form side-to-side contacts distant from the disk (Emdad et al. 2001). Following left ventricular overload, Formigli et al. observed a short-lived increase in Cx43 protein expression after creation of an aorto-caval fistula which decreased at day 7, suggesting that the up-regulation of Cx43 gap-junctional protein

may represent an immediate and transient compensatory response in the early stages of hypertrophic response (Formigli *et al.* 2003). Fialová et al. found an increase in Cx43 at the lateral cell membrane surface, particularly in spontaneous hypertensive rats (SHR) (Fialova *et al.* 2008). Of note, induction of Cx43 expression was observed of SHR fed with n-3 polyunsaturated fatty acids (n-3 PUFA) (Mitasikova *et al.* 2008). An interesting and in human studies widely unaddressed issue is the gender difference in Cx43 expression which was observed in left ventricles of aging rats (Tribulova *et al.* 2005).

Only few data are available in humans. In left ventricular biopsies of patients with hypertrophic and ischemic heart disease, Cx43 was found to be reduced (Peters *et al.* 1993). However, it appears that chronic ischemia or the presence of ventricular scars rather than cellular hypertrophy itself was responsible for these changes (Kaprielian *et al.* 1998; Peters 1996; Smith *et al.* 1991). End-stage heart failure was associated with a decrease of Cx 43 without respect of the etiology of cardiomyopathy (Kostin *et al.* 2003). Our data in humans with chronic pressure-overload with mild versus severe LVH suggest that the development of severe hypertrophy occurs without any relevant changes in Cx43 expression.

Changes in Cx43 expression have been postulated to be a mediator of electrical remodeling (Formigli *et al.* 2003; Saffitz *et al.* 1999; Severs *et al.* 2001). Decreased Cx43 expression or abnormal Cx43 distribution is associated with ventricular arrhythmias (Peters and Wit 2000). Disorganization of Cx43 was demonstrated at the border zone of myocardial infarcts in dogs and rodents, with a reduction of Cx43 at the intercalated disks and an increase of Cx43 at the lateral cell border (Matsushita *et al.* 1999; Peters *et al.* 1997). This gap junction remodeling may be associated with abnormal conduction representing a substrate for ventricular arrhythmias (Matsushita

et al. 1999; Peters *et al.* 1997; Peters and Wit 2000). Dupont *et al.* and De Mello described markedly decreased levels of Cx43 mRNA and protein in the left ventricle of patients with end-stage heart failure due to ischemic heart disease and idiopathic dilated cardiomyopathy, conditions associated with a very high risk of sudden cardiac death due to malignant ventricular arrhythmias (De Mello 1999; Dupont *et al.* 2001). Apart from disturbances in gap junction organization related to infarction, abnormal patterns of gap junction distribution are prominent in primary hypertrophic cardiomyopathy in humans, a condition associated with enhanced arrhythmic tendency (Sepp *et al.* 1996). Conversely, in our patients with secondary LVH, the analysis of multiple sections in large biopsies of subendocardial tissue did not reveal similar alterations in Cx43 distribution. Thus, secondary LVH does not seem to be associated with a potentially arrhythmogenic electrical remodeling. Indeed, the majority of patients with secondary ventricular hypertrophy survive without ventricular arrhythmias. In patients with aortic stenosis, the risk of arrhythmias and sudden cardiac death increases only late in the evolution of the disease, when the aortic stenosis becomes critical. At that stage, myocardial ischemia due to a mismatch between LVH and blood supply might be the main trigger for arrhythmias.

The absence of controls without any structural heart disease is a limitation of the study as of all such human studies because of ethical considerations. Patients without structural heart disease, or who suffer from mitral stenosis, are unlikely to undergo cardiac surgery. Moreover, only the chronic effect of pressure or volume overload on connexin expression can be analysed. Despite of these limitations the important difference in the degree of LVH as well as the difference in the cause of LVH provides us with important conclusions.

In conclusion, severe LVH due to pressure overload is not associated with detectable changes in expression or spatial distribution of Cx43 in comparison with mild LVH, or with LVH due to volume overload. Thus, at a chronic stage, the increase of cellular volume in LVH may not be associated with remodeling of Cx43 mediated cell-to-cell communication in humans.

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Table 1. Clinical characteristics of patients according to the different groups.

	MR, LVH (n=5)	AS, mild LVH (n=7)	AS, severe LVH (n=9)	Significance (p)
Age, y	75 ± 11	70 ± 7	73 ± 8	n/s
Gender m/f	3/1	3/4	5/4	n/s
BMI, g/m^2	23 ± 3	29 ± 3	26 ± 3	n/s
LVMI, g/m^2	162 ± 22	121 ± 5	179 ± 57	<0.01
LVEF, %	71 ± 9	62 ± 13	67 ± 8	n/s
Mean transaortic gradient (mmHg)	n/a	37 ± 4	47 ± 5	n/s
Arterial hypertension	3	3	7	n/a

MR, mitral regurgitation; LVH, left ventricular hypertrophy; AS, aortic stenosis; BMI, body mass index; LVEF, left ventricular ejection fraction; n/s, not significant; n/a, not applicable.

Figure legends

Figure 1a. Representative bands from western blot for Cx43 protein detection in left ventricular biopsies from different patients of the three groups: patients with volume overload due to mitral regurgitation, patients with aortic stenosis and mild left ventricular hypertrophy, and patients with aortic stenosis and severe left ventricular hypertrophy.

Figure 1b. Quantification of Cx43 protein expression in relation to β-tubulin expression by band pixel density analysis from scanned western blots for Cx43 protein detection in left ventricular biopsies from the three different groups.

Figure 2. Left ventricular sections were immunofluorescence stained for Cx43 (representative sections of the three groups). A: patient with volume overload due to mitral regurgitation. B: patient with aortic stenosis and mild left ventricular hypertrophy. C: patient with aortic stenosis and severe left ventricular hypertrophy. Cx43 (green fluorescence) is present at intercalated disks, which are seen in transverse orientation, whereas only little expression is found at the lateral cell border. Overall, Cx43 immunostaining is prominent and its distribution is similar in all groups of patients, as assessed by morphologic microscopic evaluation. Non-specific red autofluorescence of cardiomyocytes and erythrocytes is noted.

Figure 3. Real time quantitative PCR analysis of mRNA extracted from the left ventricle of the three groups of patients. Relative quantity of Cx43 mRNA expression is calculated in relation to the rRNA, and mRNA quantity of the constitutively

1 expressed house-keeping genes 18S, and GAPDH, respectively, as a measure of
2 total input RNA. There are no significant differences between the three groups.
3

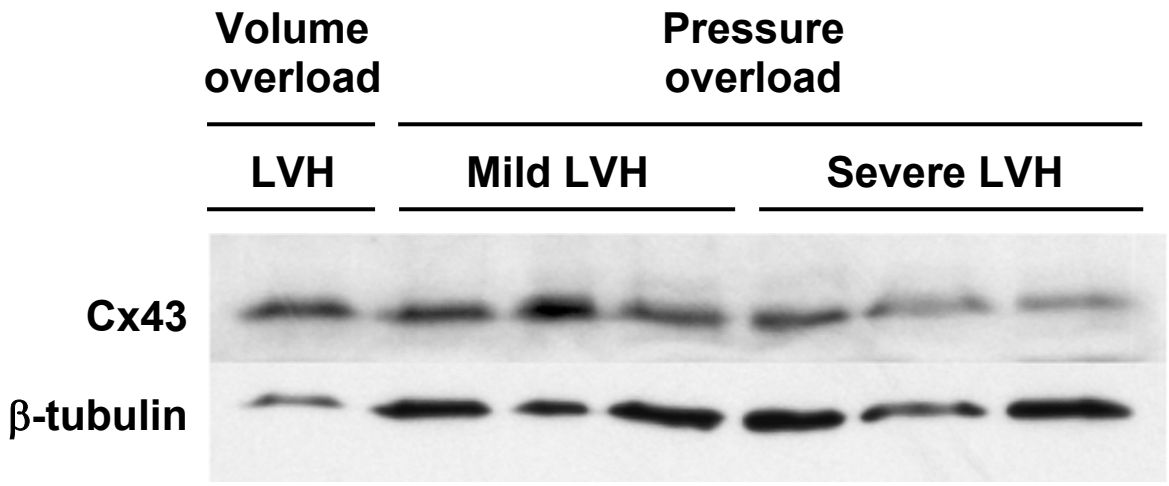
Volume overload
Pressure overload
LVH
Mild LVH
Severe LVH
Cx43
β-tubulin

Figure 1a

1

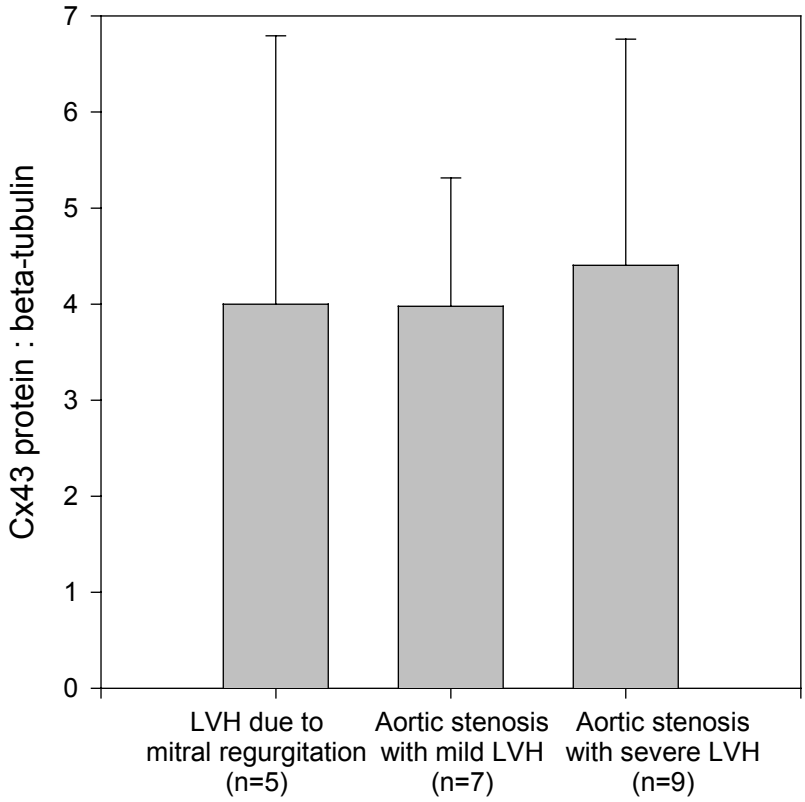
Cx43 protein : beta-tubulin
0
1
2
3
4
5
6
7
LVH due to mitral regurgitation (n=5)
Aortic stenosis with mild LVH (n=7)
Aortic stenosis with severe LVH (n=9)

Figure 1b

1

2

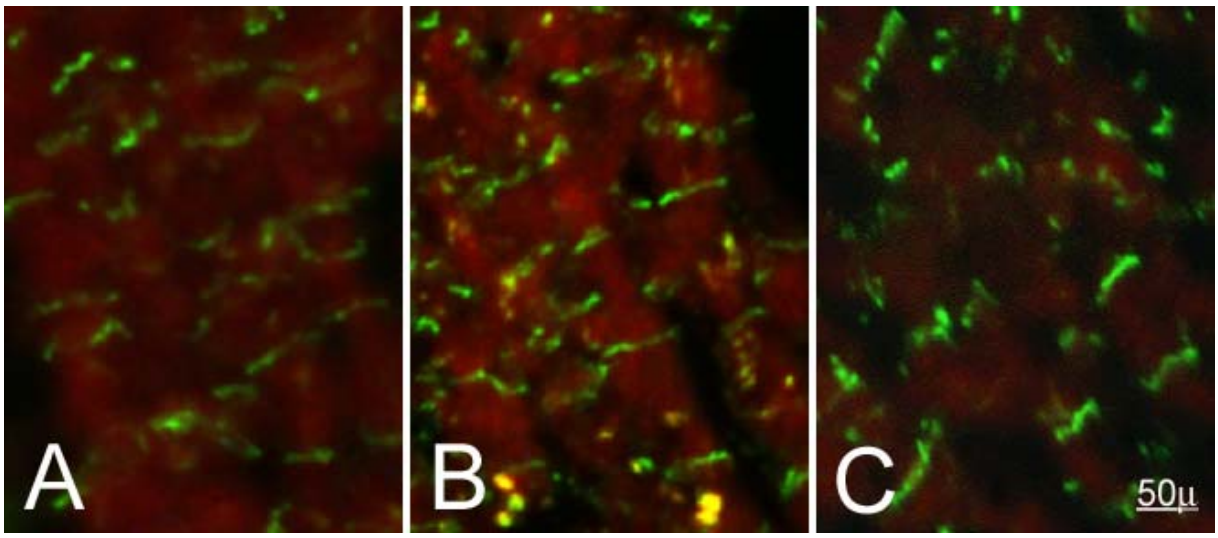

Figure 2

1

2

3

4

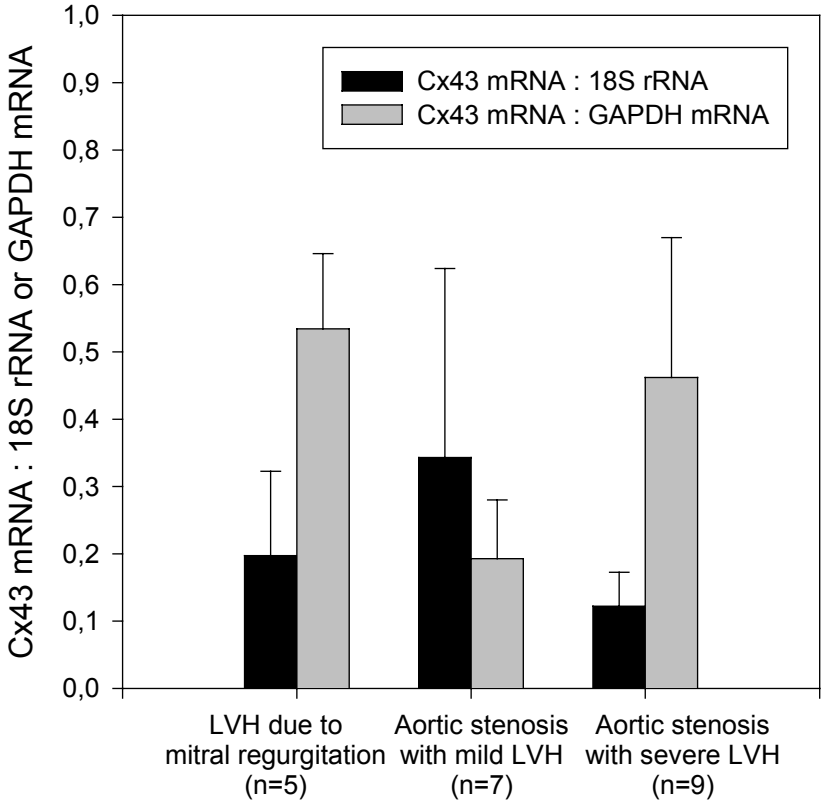

Figure 3