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Original Article

Immunohistochemical evaluation of cardiac connexin43 in rats exposed to low-frequency noise

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Abstract: Introduction: Low-frequency noise (LFN) leads to an abnormal proliferation of collagen and development of tissue fibrosis. It has been shown that myocardial fibrosis in association with gap junction remodeling occurs in several cardiac diseases and can be implicated in the development of ventricular tachyarrhythmias. We previously reported a strong development of myocardial fibrosis induced by LFN in rats but it is not known whether LFN induces any modification on cardiac connexin43 (Cx43). Objectives: The aim of this study was to evaluate modifications on cardiac Cx43 induced by LFN in Wistar rats. Methods: Two groups of rats were considered: A LFN-exposed group with 10 rats submitted continuously to LFN during 3 months and a control group with 8 rats. The hearts were sectioned from the ventricular apex to the atria and the mid-ventricular fragment was selected. The immunohistochemical evaluation of Cx43 was performed using the polyclonal antibody connexin-43m diluted 1:1000 overnight at 4°C. Quantifications of Cx43 and muscle were performed with the *image J software* and the ratio Cx43/muscle was analyzed in the left ventricle, interventricular septum and right ventricle. Results: The ratio Cx43/muscle was significantly reduced in LFN-exposed rats (p=0.001). The mean value decreased 46.2%, 22.2% and 55.6% respectively in the left ventricle (p=0.008), interventricular septum (p=0.301) and right ventricle (p=0.004). Conclusions: LFN induces modifications on cardiac Cx43 in rats. The Cx43 reduction observed in our study suggests that LFN may induce an arrhythmogenic substrate and opens a new investigational area concerning the effects of LFN on the heart.

Keywords: Low-frequency noise, connexin43, gap junction, intercalated disks, ventricular myocardium

Introduction

Low-frequency noise (LFN) leads to pathological changes in the extracellular matrix, characterized by an abnormal proliferation of collagen and the development of tissue fibrosis, in the absence of an inflammatory process [1-7]. We previously reported a significant fibrotic development in ventricular myocardium of rats exposed to LFN [8] and an increase of perivascular fibrosis in the arterial coronary vessels after exposure to industrial noise which is rich in LFN components [9], but it is not known whether LFN induces modifications on the electrophysiological *milieu*.

Gap junctions are composed by proteins known as connexins, form the intercellular pathway for electrical impulse transmission and are determinants in the genesis of cardiac arrhythmias. Changes on gap junctional connexin43 (Cx43) have been implicated in ventricular remodeling and development of arrhythmias in several cardiac diseases [10-16]. Additionally, experimental studies provided evidence that a reduction of Cx43 expression is critical to increase the propensity for ventricular tachyarrhythmias [17-22].

As LFN induces the development of myocardial fibrosis [8] and perivascular fibrosis [9] and taking into account that the presence of interstitial fibrosis in association with a decrease of Cx43 seems to have arrhythmogenic consequences [23-25], the importance of quantifying this protein is crucial to establish the occurrence of a morphological arrhythmogenic substrate induced by LFN. Thus, the aim of this study was to

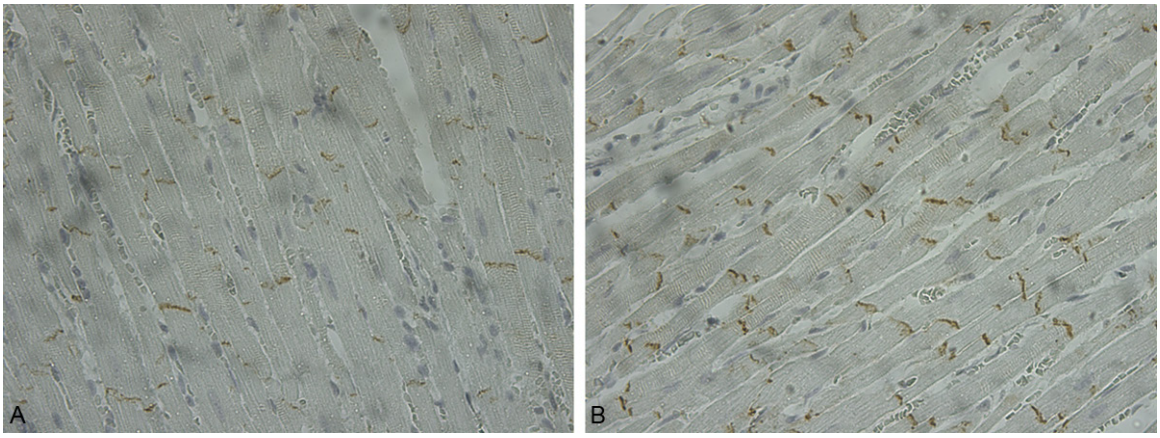

Figure 1. Immunostained connexin43 observed at the intercalated disks in a section taken from the left ventricle of a LFN-exposed rat (A) and control rat (B) (x 400).

evaluate modifications on cardiac Cx43 induced by LFN in Wistar rats.

Materials and methods

Eighteen adult Wistar rats from a Spanish breeder (Charles River Laboratories España, SA, Spain) were studied. The animals were treated in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes and with the Portuguese legislation for the same purpose. Ten rats were continuously exposed to LFN for a period of three months. The control group of 8 rats was kept in a silent environment. All the animals were kept in cages, fed standard rat food and had free access to water.

The sound signal was emitted by an analog noise generator and the noise level was the same as previously reported [26].

The hearts were fixed in 10% buffered formalin, transversely sectioned from the ventricular apex to the atria and the mid-ventricular fragment was selected for the study. The samples were incubated with polyclonal antibody connexin-43m (GJA1) diluted 1:1000 overnight at 4°C for immunohistochemical analysis.

The histological images were acquired with an optical microscope using 400 x magnifications. In each section the optical fields were selected from the left ventricle, the interventricular septum and the right ventricle. Criteria used to select each field were defined by the myocardium portions containing the highest visualization of Cx43 immunostained intercalated disks. A total of 146 optical fields were selected from all the anatomical components, by three observers, under blinded assessment, and analyzed using the *Image J software* that gives a quantification based on the image color analysis. The signal intensity threshold value of 140 on the 0 – 255 scale was identified to distinguish Cx43 from other structures. All areas with signal intensity between 0 and 140 were considered gap junctions at the intercalated disks and the following parameters were measured: 1- Cx43, 2- muscle. Then a ratio of Cx43/muscle was calculated.

Data are presented as mean ± SD. Comparisons among groups simultaneously for the three anatomical regions were performed by One-Way MANOVA, while comparisons *in totum* were performed using a t-test for independent samples. Statistical tests were applied at the 5% level of significance.

Results

The histological observation showed immunostained Cx43 at the intercalated disks and examples of sections from LFN-exposed and control rats are shown in **Figure 1**. In general, less immunoreactive particles were observed among the samples of LFN exposed rats.

The ratio Cx43/muscle in each anatomical region is shown in **Table 1** and is graphically depicted in **Figure 2**. The ratio Cx43/muscle *in totum* is also shown in **Table 1**.

Table 1. Ratio Cx43/muscle in each anatomical region and *in totum* in LFN-exposed (n=10) and control (n=8) animals

Anatomical region	Group	Mean	Std. Deviation	Percent decrease with LFN exposure	p
LV	exposed	0.014	0.006	46.2%	0.008
	control	0.026	0.008		
IVS	exposed	0.014	0.006	22.2%	0.301
	control	0.018	0.008		
RV	exposed	0.016	0.012	55.6%	0.004
	control	0.036	0.012		
In totum	exposed	0.015	0.007	43.3%	0.009
	control	0.026	0.009		

LV = Left Ventricle; IVS = Interventricular Septum; RV = Right Ventricle; LFN = Low-Frequency Noise.

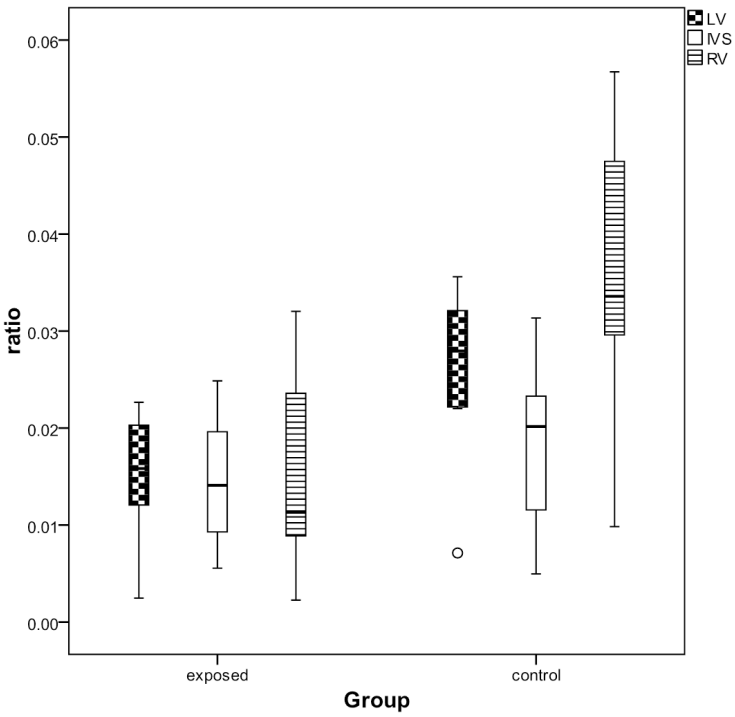

Figure 2. Ratio Cx43/muscle in the left ventricle (LV), interventricular septum (IVS) and right ventricle (RV) in LFN exposed and control animals. A significant reduction was observed in exposed animals in the left ventricle (p=0.008) and in the right ventricle (p=0.004) but not in the interventricular septum (p=0.301).

The mean values of cardiac muscle were not significantly different between the exposed and control animals in any of the anatomical regions considered in this study (p≥0.664).

The total amount of Cx43 was significantly reduced in the left ventricle (p=0.011) and in the right ventricle (p=0.009) of LFN-exposed animals when compared to controls. No differences were detected between the two groups concerning the total amount of Cx43 in the interventricular septum (p=0.237).

The ratio Cx43/muscle *in totum* decreased 43.3% among the LFN-exposed rats (p=0.009). Multivariate comparisons over the three anatomical regions showed significant differences between groups (p=0.001) with a decrease of 46.2%, 22.2% and 55.6% respectively in the left ventricle (p=0.008), in the interventricular septum (p=0.301) and in the right ventricle (p=0.004).

Discussion

As far as we know this is the first study concerning the evaluation of cardiac Cx43 in rats submitted to LFN.

In humans, gap junction remodeling has been studied in several pathologies and arrhythmias [10-16, 27, 28]. The loss of Cx43 expression has been shown to be a key for the development of an arrhythmic anatomic substrate in chronically hypertrophied myocardium [15]. Modifications in gap junction organization and on Cx43 expression contribute to conduction disturbances and development of arrhythmias in myocardial infarction [27], non-ischemic dilated cardiomyopathy [12, 13], cardiac heart failure [11] and valvular heart disease [14].

In several studies, a reduction of Cx43 from 30 to 50% occurs in ventricular remodeling [14, 23, 29], but changes in gap junction expression alone are presumably not sufficient for conduction slowing and enhanced arrhythmogenicity, apparently because there is a large conduction reserve [30].

Meanwhile, it is known that an increase of the intercellular collagen deposits may lead to

anisotropic reentry [23, 24], and a strong enhancement of arrhythmogenic vulnerability can be attained by the association of increased fibrosis with a 50% reduction of Cx43 expression [31].

As LFN induces the development of interstitial myocardial fibrosis [8] and perivascular fibrosis [9] we hypothesized that the finding of significant modifications on gap junctions after LFN exposure could lead to a morphological arrhythmogenic substrate.

In our study the measurement of Cx43 was performed in equivalent tissue mass among exposed and control animals and showed a significant reduction in rats exposed to LFN. The modification was evident at the free ventricular wall but not in the interventricular septum suggesting that this anatomical region could be more protected against the effects of LFN. However, this does not discard the possibility of a strong alteration of the electrophysiological *milieu*. In fact, the observed statistical powers of 99% for the multiple comparisons between groups and in excess of 80% for the comparisons between groups regarding the left and right ventricles are noteworthy, in view of the sample dimension, suggesting the marked effects of LFN exposure.

Taking into consideration the universal existence of LFN in modern societies and having in mind the difficulties to explain some ventricular tachyarrhythmias without structural heart disease, the hypothesis of idiopathic ventricular fibrillation as being also a consequence of gap junction remodeling mediated through the effects of LFN, should not be despicable. On the other hand, in patients with already known specific cardiac disorders, a reduction of gap junctions by the exposure to LFN makes the development of arrhythmic events possible, carrying out an adverse prognosis.

We still do not know the mechanisms underlying the fibrotic development we previously reported in rats exposed to LFN [8] or to industrial noise [9] as well as the mechanism of the Cx43 alteration observed in this study. Nevertheless, the Cx43 remodeling has been linked to intrinsic factors occurring on biosynthesis at transcriptional or posttranscriptional phase [32, 33]. As connexin43 is degraded through lysosomal or proteasomal pathway [34, 35] one can also speculate that the Cx43 reduction observed in this study can be related to an activation of these pathways. Theoretically, LFN acting as an external mechanical force could also lead to activation of protein kinases which might modify the level of Cx43 phosphorylation [36, 37].

Regardless of the mechanism how LFN induces loss of Cx43, our results suggest that the significant reduction of this protein can lead to electrophysiological modifications. The occurrence of a significant myocardial and periarterial fibrosis induced respectively by LFN and industrial noise reported earlier by our group [8, 9], together with a possible gap junction remodeling observed in this study, makes the development of a morphological arrhythmogenic substrate by LFN possible. Further experimental and clinical studies are needed to evaluate the functional and arrhythmic consequences. With this study we put forward the hypothesis of a link between LFN and ventricular tachyarrhythmias, opening a new investigational area concerning the effects of LFN on the heart.

In conclusion, we can state that low frequency noise induces modifications on cardiac connexin43 in rats. The connexin43 reduction observed in our study can contribute to a morphological arrhythmogenic substrate.

Disclosure of conflict of interest

None.

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