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Ana Maria Pereira Alves Lousinha Kahlbau

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New Observations on Cardiac Morphological Changes induced by Low Frequency Noise and Infrasound in Rats

Tese de Candidatura ao grau de Doutor em Ciências Médicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Prof. Doutor Eduardo José Pereira Antunes, professor convidado do Instituto Universitário Egas Moniz, Caparica.

Co-orientadora – Professora Doutora Maria João Oliveira, professora associada com agregação do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

To my family

“One day man will have to fight noise as fiercely as cholera and plague”

Robert Koch, 1910

List of articles included in this thesis

Lousinha A, Antunes E, Borrecho G, Oliveira MJ, Brito J, Martins dos Santos J (2015) Histomorphometric evaluation of the small coronary arteries in rats exposed to industrial noise. *Int J Mol Sci* 16:1095-1104.

Lousinha A, Oliveira MJR, Borrecho G, Brito J, Oliveira P, Oliveira de Carvalho A, Freitas D, Águas A, Antunes E (2018) Infrasound induces coronary perivascular fibrosis in rats. *Cardiovasc Pathol* 37:39-44.

Lousinha A, Pereira G, Borrecho G, Brito J, Oliveira P, Oliveira de Carvalho A, Freitas D, Oliveira MJR, Antunes E. Atrial fibrosis and decreased connexin 43 in rat hearts after exposure to high-intensity infrasound. Submitted for publication in Exp Mol Pathol, Dec 2019.

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ACRONYMS AND ABBREVIATIONS

CAB – Chromotrope-Aniline Blue

Cx - Connexin

dB - Decibel

ECM – Extracellular Matrix

hi-IFS – High-Intensity Infrasound

Hz – Herz

IFS – Infrasound

IN – Industrial Noise

LFN – Low Frequency Noise

L/W – Lumen-to-Vessel Wall

LV – Left Ventricle

Pa – Pascal

RV – Right Ventricle

WHO – World Health Organization

W/P – Vessel Wall-to-Perivascular Tissue

ABSTRACT

Introduction: Noise is an important environmental and occupational risk factor and human exposure to this aggressor can induce systemic damage. The relative contribute of noise intensity, frequency content, mean and dB peak level, pattern or duration of the exposure, that may be responsible for inducing this aggression is not established. Previous investigations suggest that high-intensity noise, in a wide spectrum of wavelengths, from industrial noise (IN) to low frequency noise (LFN) and infrasound (IFS), can act as a physical stressor. The main morphological change induced by this type of noise is a systemic abnormal proliferation of connective tissue affecting several organs and tissues, such as gastric mucosa, lung parenchyma, tracheal epithelia, adrenal cortex, parotid gland, lymphatic and arterial vessels and the heart.

Objectives: This thesis was build up on previous research on the cardiac morphological changes induced by IN and LFN in Wistar rats and addressed important gaps in knowledge that have emerged from those studies. We evaluated: 1) whether structural changes induced by IN exposure extends to the small coronary arteries; 2) whether IFS, likewise LFN, causes morphological changes in rat coronaries and if these are influenced by treatment with an anti-inflammatory agent; 3) if the atria are vulnerable to the effects of high-intensity IFS exposure.

Material and Methods: The first series was used to evaluate the histomorphometric changes of the small coronary arteries induced by IN and included 20 rats exposed to noise with ≤500Hz and ≥90dB, during a maximum period of seven months and 20 age-matched controls. The second series included 14 adult rats divided into three groups: group A (GA)-IFS (<20 Hz, 120dB)-exposed rats for 28 days treated with dexamethasone; group B (GB)-IFS-exposed rats; group C (GC)-age-matched controls. In the third series, 72 rats, half exposed to high-intensity IFS (120dB, <20Hz) during a maximum period of 12 weeks, and half age-matched controls, were studied to assess atrial fibrosis and connexin 43 (Cx43) modifications. In all series, hearts were transversely sectioned from ventricular apex to atria and the mid-ventricle or the atria were selected for analysis. The histological images were obtained with an optical microscope (Leica® MZ6), equipped with a digital camera (Leica® DF 290HD). In the first study, a total of 634 arterial vessels (298 from IN-exposed and 336 from controls) were selected for analysis and in the second, 31 arterial vessels (GA 8, GB 10, GC 13). Perivascular and atrial fibrosis were analyzed by Chromotrope-aniline blue staining and the immunohistochemical evaluation of Cx43 was performed using the polyclonal antibody Cx43m diluted 1:1000 at 4ºC overnight. The image J software was used for measurement of the morphological parameters. The vessel caliber, thickness of the wall and perivascular dimensions were quantified and the

mean lumen-to-vessel wall (L/W) and mean vessel wall-to-perivascular tissue (W/P) ratios were calculated. For the atrial fibrosis evaluation, the areas of muscle and of interstitial fibrosis were quantified and the mean "atrial fibrosis / cardiomyocytes" ratio was calculated. To evaluate Cx43 a similar method was employed and the mean "Cx43 / cardiomyocytes" ratio was calculated. Either a two-way ANOVA model (studies 1 and 3) or Mann-Whitney and Kruskal-Wallis tests (study 2) were used to compare the groups.

Results: In the first study we found no differences between exposed and control animals in their L/W ratios ($p=0.687$) and time variations in this ratio were non-significant ($p=0.110$). In contrast, exposed animals showed lower W/P ratios than control animals ($p<0.001$), with significant time variations ($p=0.004$). In the second study, IFS-exposed rats exhibited a prominent perivascular tissue. The median L/W and median W/P ratios were 0.54 and 0.48 (GA), 0.66 and 0.49 (GB) and 0.71 and 0.68 (GC). The W/P ratio was significantly higher in GC compared with IFS-exposed animals ($p=0.001$). The difference was significant between GC and GB ($p=0.008$) but not between GC and GA. In the third study, the mean values of the "atrial fibrosis / cardiomyocytes" ratio increased to a maximum of $0.1095\pm0,04$ and $0.5408\pm0,01$, and of the "CX43 / cardiomyocytes" ratio decreased to $0.0834\pm0,03$ and $0.0966\pm0,03$, respectively in IFS-exposed rats and controls. IFS-exposed rats exhibited a higher ratio of fibrosis ($p<0.001$) and lower ratio of Cx43 ($p=0.009$).

Conclusions: Exposure to IN increases the perivascular tissue of rat small coronary arteries, with significant development of periarterial fibrosis. Chronic exposure also causes thickening of the small coronary vessel wall. High pressure level IFS exposure induces coronary perivascular fibrosis in rats and the existence of an underlying inflammatory mechanism should be considered. Infrasound exposure affects the atria of rat hearts, with an increase of interstitial fibrosis and a decrease of Cx43. The effects of exposure to LFN and IFS seems to be independent of its pattern but chronic exposure can elicit additional structural changes, not observed in the acute setting. We propose that both sound frequency and pressure level are key factors to explain the toxicological effects induced by noise and should be considered in future research.

RESUMO

Introdução: O ruído é um importante fator de risco ambiental e ocupacional e a exposição humana a este agressor pode induzir danos sistémicos. A contribuição relativa da intensidade do ruído, frequência, pressão sonora média e máxima, padrão ou duração da exposição, responsável por induzir essa agressão não está estabelecida. Investigações prévias sugerem que o ruído de alta intensidade, dentro de um amplo espectro de comprimentos de onda, desde ruído industrial (RI) ao ruído de baixa frequência (RBF) e infrassom (IFS), pode atuar como fator de stress físico. A principal alteração morfológica induzida por este tipo de ruído é a proliferação sistémica anormal do tecido conjuntivo afetando vários órgãos e tecidos, como a mucosa gástrica, o parênquima pulmonar, o epitélio traqueal, o córtex suprarrenal, a glândula parótida, os vasos linfáticos e arteriais e o coração.

Objetivos: A presente tese foi desenvolvida a partir de investigações anteriores sobre as alterações morfológicas cardíacas induzidas pelo RI e RBF em ratos *Wistar* e abordou importantes lacunas no conhecimento com origem nesses estudos. Avaliámos: 1) se as alterações estruturais induzidas pela exposição ao RI se estendem às pequenas artérias coronárias; 2) se o IFS, tal como o RBF, causa alterações morfológicas nas artérias coronárias e se estas são influenciadas pelo tratamento anti-inflamatório; 3) se as aurículas são vulneráveis aos efeitos da exposição ao IFS com alta intensidade.

Material e Métodos: A primeira série foi utilizada para avaliar as alterações histomorfométricas das pequenas artérias coronárias induzidas por RI e incluiu 20 ratos expostos a ruído com ≤500Hz e ≥90dB, por um período máximo de sete meses, e 20 controlos com a mesma idade. A segunda série incluiu 14 ratos adultos divididos em três grupos: grupo A (GA) com ratos expostos ao IFS (<20Hz, 120dB) durante 28 dias tratados com dexametasona; grupo B (GB) com ratos expostos ao IFS; grupo C (GC) com controlos da mesma idade. Na terceira série, 72 ratos, metade expostos ao IFS (<20Hz, 120dB), período máximo de 12 semanas, e controlos emparelhados por idade, foram estudados para avaliar a fibrose auricular e as modificações da conexina 43 (Cx43). Em todas as séries, seccionaram-se transversalmente os corações do ápice ventricular às aurículas e selecionaram-se para análise o ventrículo médio ou a aurícula. As imagens histológicas foram obtidas com um microscópio ótico (Leica® MZ6) equipado com câmara digital (Leica® DF 290HD). No primeiro estudo, selecionaram-se para análise 634 vasos arteriais (298 de ratos expostos ao RI e 336 de controlos) e no segundo, 31 vasos arteriais (GA 8, GB 10, GC 13). A fibrose perivascular e auricular foi analisada com coloração azul de anilina (CAB) e realizou-se a avaliação

imunohistoquímica de Cx43 com anticorpo policlonal Cx43m diluído 1:1000 a 4ºC. O software *image J* foi usado para medição dos parâmetros morfológicos. O calibre do vaso, espessura da parede e dimensões perivasculares foram quantificados e calcularam-se as médias dos rácios lúmen - parede do vaso (L/W) e parede do vaso - tecido perivascular (W/P). Para avaliação da fibrose auricular, quantificaram-se as áreas de músculo e fibrose intersticial e calculou-se a média do rácio "fibrose auricular / cardiomiocitos". Para avaliar a Cx43 foi utilizado um método semelhante e calculou-se a média do rácio "Cx43 / cardiomiocitos". Para comparar os grupos utilizaram-se a análise de variância com dois fatores (estudos 1 e 3) ou os testes de Mann-Whitney e Kruskal-Wallis (estudo 2).

Resultados: O primeiro estudo não mostrou diferenças entre os rácios L/W dos animais expostos e respetivos controlos ($p=0,687$) e as variações temporais não foram significativas ($p=0,110$). Por outro lado, os animais expostos apresentaram menores rácios W/P que os controlos ($p<0,001$), com variações significativas no tempo ($p=0,004$). No segundo estudo, ratos expostos ao IFS exibiram um tecido perivascular proeminente. As medianas dos rácios L/W e W/P foram de 0,54 e 0,48 (GA), 0,66 e 0,49 (GB) e 0,71 e 0,68 (GC). O rácio W/P foi maior no GC em comparação com animais expostos ao IFS ($p=0,001$). A diferença foi significativa entre os GC e GB ($p=0,008$), mas não entre os GC e GA. No terceiro estudo, os valores médios do rácio "fibrose auricular / cardiomiocitos" aumentaram para um máximo de 0,10950±04 e 0,54080±01, e do rácio "CX43 / cardiomiocitos" diminuíram para 0,08340±03 e 0,09660±03, respetivamente em ratos expostos ao IFS e controlos. Os primeiros apresentaram uma proporção maior de fibrose ($p<0,001$) e menor proporção de Cx43 ($p=0,009$).

Conclusões: A exposição ao RI aumenta o tecido perivascular das pequenas artérias coronárias de ratos, com fibrose periarterial significativa. A exposição crónica também causa espessamento da parede desses vasos. A exposição ao IFS com pressão sonora elevada induz fibrose coronária perivascular em ratos, devendo considerar-se a existência de um mecanismo inflamatório subjacente. A exposição ao IFS afeta as aurículas dos corações dos ratos, com aumento da fibrose intersticial e diminuição da Cx43. Os efeitos da exposição ao RBF e IFS parecem ser independentes do padrão de exposição, mas a exposição crónica pode provocar alterações estruturais adicionais, não observadas na exposição aguda. Propomos que quer a frequência quer a pressão sonora são fatores fundamentais para explicar os efeitos toxicológicos induzidos pelo ruído, devendo ser consideradas em investigações futuras.

1. INTRODUCTION

1.1 Basic acoustics, low frequency noise and infrasound

Sound is the term used to describe any pressure variation that propagates through a medium (which may be a gas, a liquid or a solid) and that can be sensed by the auditory system of a human being [1]. Noise and sound are the same from a physics standpoint, but the reaction to perception varies between people, depending on the cognitive environment in which detection occurs and ultimately leads to a designation of noise as an undesired sound [2-3].

In order to facilitate understanding of the studies included in this thesis, we address here two important characteristics of sound: **frequency** and **pressure level**.

The **frequency** of the sound, measured in Herz (Hz), corresponds to the number of pressure variations per second [1]. For acoustic and sound measurement purposes, these pressure variations travel through dry air at room temperature (20ºC) at a speed of approximately 343 meters per second. The speed and frequency of a sound permit to calculate the distance between two contiguous pressure peaks, named **wavelength** and represented by the greek letter λ, using the following formula:

$$\text{Wavelenght } (\lambda) = \frac{\text{Speed of sound (meters)}}{Frequency\ (Hz)}$$

Thus, the frequency and the wavelength of a sound are inversely proportional. The vast majority of sounds are made up of different frequencies and have complex waveforms, as opposed to pure sinusoidal tones consisting of a single frequency [1].

Any vibration of a person's eardrum in the audible frequency range – 20 to 20000 Hz being the most accepted interval worldwide - that results from an additional variation in atmospheric pressure at the ear is perceived as sound. That incremental variation above and below atmospheric pressure is called **sound pressure** and is measured in units of Pascal (Pa). Since measuring sound in Pa would imply extremely large and unmanageable measurement ranges (for example, the lowest sound a healthy human ear can perceive has an amplitude of 20 millionths of a Pascal – 20 μPa – which is five billion times less than normal atmospheric pressure), a logarithmic scale was adopted, using the **Decibel (dB)** as a unit of sound pressure level. The hearing threshold of 20 μPa is used as the reference level and is defined as 0 dB [1].

Low frequency noise (LFN) and **infrasound (IFS)** are conventionally defined as sound with frequencies below 200 and 20 Hz, respectively (figure 1).

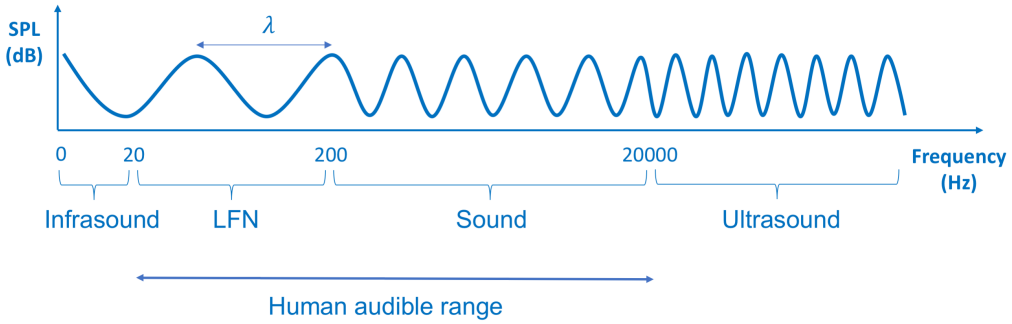

Figure 1. Classification of sound according to frequency and the conventionally accepted human audible range. LFN – low frequency noise, SPL – sound pressure level.

The definition of IFS as an inaudible sound can be misleading, as the lower limit of the audio frequency range of human hearing is usually given as 20 Hz but humans can perceive IFS if the sound pressure level is sufficiently high [4]. Watanabe and Møller [5] documented human hearing at frequencies as low as 4Hz, as shown in table 1.

Table 1: Human hearing thresholds using pure sinusoidal tones as sound stimuli

Freq (Hz)	**4**	**8**	**10**	**12,5**	**16**	**20**	**25**	**31,5**	**40**	**50**	**63**	**80**	**100**	**125**
Level (dB)	107	100	97	92	88	79	70	61	51	46	36	33	27	25

(Adapted from Watanabe and Møller. *J. Low Frequency Noise Vibrat.* 1990; 9: 106-115)

In the frequency range of IFS, studies comparing the auditory sensitivity of different animal species have shown broad differences between them. For instance, rats have poorer infrasonic hearing than humans, taking into account different sound pressure levels [6]. Nonetheless, rats can perceive high-intensity (≥110dB) IFS vibrations, as they elicit active avoidance reactions under experimental conditions [7].

Regarding sound propagation, an obstacle in the sound path will cause its reflection, absorption or transmission through the object. In general, these three processes depend on

the wavelength of the sound. Low frequency noises, including infrasound, by having longer wavelengths, are presumably transmitted more significantly through the body wall [8-10].

Low frequency noise and IFS are present everywhere, from natural occurrences, such as wind, ocean waves or earthquakes, to artificial sources that include industrial installations and low-speed machinery, like diesel engines and wind turbines [9] (figure 2).

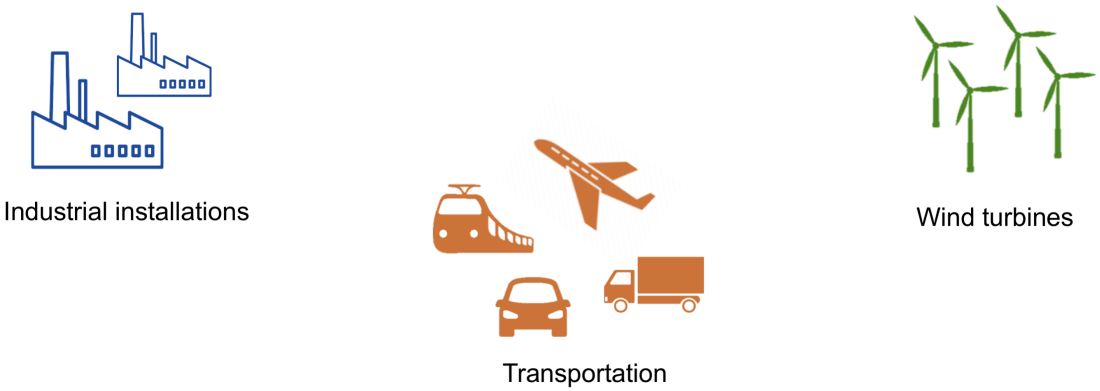

Figure 2. Artificial sources of low frequency noise and infrasound.

The principal instrument used for measuring sound pressure levels in the environment and in the occupational setting is the Sound Level Meter, usually consisting of a microphone, a processing section and a read-out display [1]. The microphone converts the sound signal to an equivalent electrical signal and different types of processing may be performed on that signal. The signal may pass, for example, through a weighting network. Since the human ear is not equally sensitive to all sound frequencies, noise levels at maximum human sensitivity are factored more heavily into some measurements using frequency weighted filters. There are several but the **A-weighting filter** is the most widely used in environmental studies. In spite of the fact that it covers the full frequency range of 20 to 20000 Hz, the shape approximates to the frequency sensitivity of the human ear, gradually reducing the significance of frequencies below 1000 Hz. As a consequence, LFN and IFS cannot be correctly evaluated using the conventional A-filters and are, therefore, misrepresented in these studies [10].

1.2 The Wistar rat: anatomy and histology of the heart and cardiovascular system

The rat heart has four chambers, the left and right ventricles and the atria [11]. The left ventricle has a thick wall that continues as the interventricular septum, the right ventricle has a thinner wall and both atria are thin walled.

Two valves, the tricuspid valve on the right and the mitral valve on the left, separate the ventricles from the atria. Located on the ventricular outflow tract, at the root of each great artery, are the semilunar valves, aortic and pulmonary.

The blood supply to the heart is provided by two coronary arteries, left and right. In rats, coronary arteries are buried more deeply in the myocardium, while in humans they lie usually epicardially [12].

In rat heart the pulmonary veins join outside the left atrium and there is just one opening into that chamber, while in humans there are four pulmonary veins that enter the left atrium by four separate orifices.

Specific descriptions of the histology of the vasculature of the rat are lacking but this should not be seen as a disadvantage, as the structure of the blood vessels is very similar to that of humans [13].

Arteries are divided into the elastic arteries, such as the aorta, and the muscular arteries, such as the smaller arteries seen in histological sections of the heart. In both types, the arterial wall consists of three tissue layers: *intima* (inner coat comprising the endothelium, the subendothelial connective tissue and the internal elastic lamina), *media* (in muscular arteries, this is a thick layer of smooth muscle interwoven with collagen, reticulin and elastic fibres) and *adventitia* (outer layer of connective tissue comprising mainly collagen).

Arterioles are the terminal branches of the arterial system and those with larger caliber have a relatively thick *media* layer.

Concerning the histology of the heart, the walls of the atria and ventricles are made up of an endocardium, a myocardium and an epicardium [14].

The endocardium comprises the endothelium that lines the chambers of the heart, a subendothelial layer of connective tissue and a subendocardial layer, also of connective tissue,

but containing some elastic fibres. In the human heart, the endocardium is thicker in the atria than in the ventricles.

The myocardium is made up of chains of cardiac muscle cells interspersed with connective tissue forming myocardial fibres, in which run the blood vessels and nerve fibres that supply the muscle cells. Myocardial cells are cylindrical and branch frequently, exhibiting an irregular arrangement. Between the ends of two cells is an intercalated disc, with a stepped appearance when seen at light microscopy. The classic definition of the intercalated disc includes three main structures: the desmosome (which functions as a cell anchor), the adherens junction (which provides cell strength) and the gap junction (which couples cells electrically and metabolically) [15].

Gap junctions are composed of subunit proteins called connexins. In the heart there are three main connexin (Cx) isoforms: Cx43, Cx40 and Cx45. Connexin 43 is the most abundant and is distributed among atrial and ventricular cardiomyocytes and Purkinje fibers; Cx40 is mainly expressed in the atrial myocytes; and Cx45 is mainly expressed in the specialized conduction system [15]. Although the distribution patterns of connexins are, to a large extent, comparable between mammals, some differences exist, as described by van Kempen *et al* [16], such as the lack of Cx40 in the atria of adult rats.

The epicardium comprises a single, superficial layer of mesothelial cells of the visceral pericardium and an underlying layer of connective tissue [13].

1.3 Effects of LFN and IFS on morphology and histopathology in experimental studies

Noise is an important environmental and occupational risk factor and it is consensual that human exposure to this aggressor can induce systemic damage, with a consequent impact on public health. The World Health Organization (WHO) Regional Office for Europe has been particularly concerned with sound pressure level (dB) limits but also acknowledges that LFN, below 200Hz, represents an environmental problem [17, 12].

Research on the impact of LFN and IFS established that they are hazardous for the human body, particularly for sound pressure levels above 120dB [8, 9]. The characteristics of strong penetration and less attenuation in long distance propagation have been proposed to explain several adverse biological effects, in experimental and epidemiological studies [9]. High intensity-LFN and IFS can induce resonance responses in body cavities [9]. The overall range

of human body resonant frequencies was found to be from 2 to 16Hz [19], which is almost the exact range of IFS. The displacement between organ and skeletal structures places biodynamic strain on the involved body tissue and it is known to reach its maximum under exposure to noise close to the body's resonant frequency. Thus, high pressure levels of LFN can act as a mechanical stressor [8, 9, 19, 20].

Noise affects both auditory and non-auditory systems of humans exposed to it [21]. The type of exposure (continuous, occasional and occupational) and its duration may trigger different responses [22, 23]. According to experimental and epidemiological studies, the non-auditory effects of noise include annoyance, sleep disturbance and psychological stress and can elicit deleterious systemic effects [8, 9, 21].

As well as humans, other animals also possess inherent specific sound frequencies in certain tissues and organs and may be susceptible to the mechanical stress prompted under exposure to high pressure levels of LFN [24]. Studies on the morphological and biological effects of exposure to different types of noise, from industrial to LFN, in animal models, showed an increased volume of connective tissue and collagen fibers in different tissues and organs, such as lung parenchima, tracheal epithelia, gastric mucosa, lymphatic vessels, arterial vessels, parotid gland and liver [25-33].

1.4 Effects on the cardiovascular system

The cardiovascular system of rodents is sensitive to LFN [34-36]. The first studies in laboratory animals were conducted three decades ago. In these studies, rats were exposed to IFS (≤16Hz at ≥90dB) for up to 45 days and ultimately developed myocardial ischemia and morphofunctional changes in the myocardium cells [37-39].

In humans, two decades ago, morphological changes were observed in the cardiac valves, consisting of thickening, calcification and/or restriction of leaflet movement and affecting predominantly the mitral valve [40]; and in the pericardium, with thickening and development of additional cell layers [41].

More recently, Pei *et al* reported IFS-induced hemodynamics, cardiac ultrastructure damage and cardiac cell apoptosis in the rat myocardium [42, 43]. The same group found that IFS dysregulates the L-type calcium currents in rat ventricular myocytes [24], and also that acute exposure to IFS induces oxidative damage of cardiomyocytes that affects a series of oxidative

damage-related proteins and genes, suggesting a complex signalling network that is evoked by this stressor [44].

Industrial noise (IN) exposure, rich in LFN, triggered the development of perivascular fibrosis around the coronary arteries of Wistar rats [45]. Low-frequency noise exposure, using the same animal, elicited the development of significant interstitial fibrosis in ventricular myocardium [46, 47]. These morphological changes were found in the absence of inflammatory cells, which could suggest a non-inflammatory process. However, the fibrotic proliferation mechanism remains unclear.

The differentiation of cardiac fibroblasts into more active myofibroblasts, a complex and highly regulated process where biochemical and mechanical factors are interdependent, is the hallmark of cardiac fibrosis [48, 49]. Although the role of mechanical factors remains elusive, cardiac fibroblasts exposed to abnormal mechanical conditions such as strain and extracellular matrix stiffness can undergo myofibroblast differentiation, leading to an abnormal accumulation of the extracellular matrix components, such as collagen, in the heart myocardium [50, 51].

1.5 Perivascular fibrosis of the small coronary arteries

Exposure to LFN with high sound pressure level (>100dB) affects medium and large-calibre blood vessels of Wistar rats, such as the aorta, inferior vena cava and femoral artery and vein [30]. With long-term exposure, the aorta and the femoral artery showed a focal thickening of the intima, disruption of the internal elastic lamina and a proliferation of smooth muscle cells in the intima. These morphological changes were not observed in the small vessels.

In the heart, the coronary artery vessels of rats exposed to industrial noise showed prominent perivascular tissue and fibrotic development [45].

The changes in the structure of coronary resistance vessels, with quantitative characterization of small coronary arteries and arterioles in the myocardium, have been extensively studied under several experimental conditions and were already extended to humans. For instance, cardiac concentric hypertrophy in hypertension is characterized by increased wall thickness in arterioles and small arteries, increased lumen to wall ratio and decreased number of capillary profiles per arteriole in cross section [52]. Volume overload-induced cardiac hypertrophy is characterized by normal coronary reserve and maximal flow and there is evidence that both

arteriolar and capillary growth is proportional to the magnitude of hypertrophy [53]. Diabetic cardiomyopathy is described as a microvascular disease, where structural changes include thickening of the vascular wall, perivascular fibrosis, capillary aneurysms and decrease in capillary density [54, 55]. In chronic renal failure, the structural abnormalities of the heart include arteriolar thickening [56], reduced capillary density [57] and interstitial fibrosis [58]. All of these structural findings share similar clinical outcomes such as myocardial ischemia, left ventricular wall stiffness, diastolic dysfunction and arrhythmogenicity [59].

We sought to investigate whether exposure to industrial noise and LFN can also induce structural changes in the small arteries and arterioles of the rat heart.

1.6 The role of inflammation as an underlying mechanism

As previously mentioned, the common finding in the noise experiments conducted by our group was the perivascular and myocardial fibrotic development in the absence of inflammatory cells [25-33, 45-47], leading to the assumption that inflammation was not involved in its pathophysiology.

Some recent studies point to a hypothetical noise-induced pathway involving inflammation, although the evidence is limited [60]. Munzel *et al* [35] developed a novel noise exposure model in mice that resulted in increased biomarkers of inflammation as well as in the invasion of the vasculature with inflammatory cells. The same group demonstrated that nighttime aircraft noise in healthy volunteers can cause endothelial dysfunction, which is partially corrected by the acute administration of vitamin C, pointing instead to increased oxidative stress as a key mechanism [61]. In humans, sleep disturbance is associated with a pro-inflammatory state [62].

In face of these findings, it became mandatory to address this matter in our investigations and to explore the potential role of inflammation as an underlying mechanism.

1.7 Atrial interstitial fibrosis and connexin 43 modifications

The immunohistochemical evaluation of cardiac Cx43 in the ventricle performed by our group showed a reduction in its concentration among LFN-exposed rats [63]. Several experimental studies demonstrated that reductions in Cx43 are associated with slowing of impulse propagation and increased vulnerability to ventricular arrhythmias [64-66]. If myocardial fibrosis also increases under exposure to LFN, as previously reported [46, 47], it is licit to assume the existence of a noise-induced morphological arrhythmogenic substrate.

With the same rationale in mind, we decided to test the hypothesis that different periods of high intensity infrasound exposure (120dB, <20Hz) can lead to atrial structural remodeling involving the development of interstitial fibrosis and modifications of Cx43 in rats.

Animal models contribute to our knowledge of arrhythmogenesis. The pathophysiological basis of most arrhythmias is not completely understood but different types of arrhythmias share similarities in their basic mechanisms [67]. For instance, despite the different underlying mechanisms proposed, the most consistently reported structural change in patients and animals with AF is atrial fibrosis [68, 69].

Since 2016, three large cohort studies showing an association between noise exposure and atrial fibrillation were published [70-72]. In two of these investigations the association reached statistical significance. Currently, there are no studies specifically addressing the pathophysiology of noise-induced atrial fibrillation.

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2. OBJECTIVES

OBJECTIVES

The analysis of the available scientific data, clinical and experimental, suggests that the main morphological change induced by low frequency noise and infrasound is a systemic abnormal proliferation of connective tissue.

It is widely accepted that noise has impact on public health but the relative contribute of its intensity, frequency content, mean and dB peak level, as well as the pattern or duration of the exposure, that may be responsible for inducing this aggression is not well understood. Concerning the characteristics of noise, public health research uses A-weighting method to measure noise and focus on sound pressure level, disregarding frequencies.

We believe that both sound frequency and intensity are key factors. As stated before, the overall range of human body resonant frequencies overlaps the frequency range of IFS. It may be assumed that animals also possess inherent specific sound frequencies in certain tissues and organs and for that reason it is important to document, using animal models, the morphological and biological effects induced by a wide spectrum of wavelengths, from industrial to LFN and IFS.

The purpose of the studies included in this thesis is to further contribute to the knowledge of the effects of LFN and IFS on the heart, addressing the following questions:

1. What are the effects of exposure to industrial noise, characterized by high pressure levels and a wide spectrum of wavelengths that includes LFN, on the morphology of small coronary arteries and arterioles in the rat heart?

2. Can high pressure level IFS (<20Hz) exposure, just as LFN, induce coronary perivascular fibrosis in rats?

3. Does the administration of an anti-inflammatory agent interfere with the development of IFS-induced perivascular fibrosis?

4. Are the effects of high pressure level IFS exposure on the heart extensible to the atria?

5. Finally, are the effects of exposure to LFN and IFS dependent on its pattern (intermittent vs continuous) or duration (weeks to months)?

In order to answer these questions, we evaluated three series of Wistar rats that were used in three consecutive studies (figure 1). In chapter 3, we present the histomorphometric evaluation of the small coronary arteries in rats exposed to industrial noise. In chapter 4, the morphological changes induced by IFS in the presence and absence of an anti-inflammatory agent are addressed. Chapter 5 focuses on the atrial remodeling in rat hearts after exposure to high intensity IFS. Finally, chapter 6 comprises the final discussion of the findings of these studies and respective conclusions.

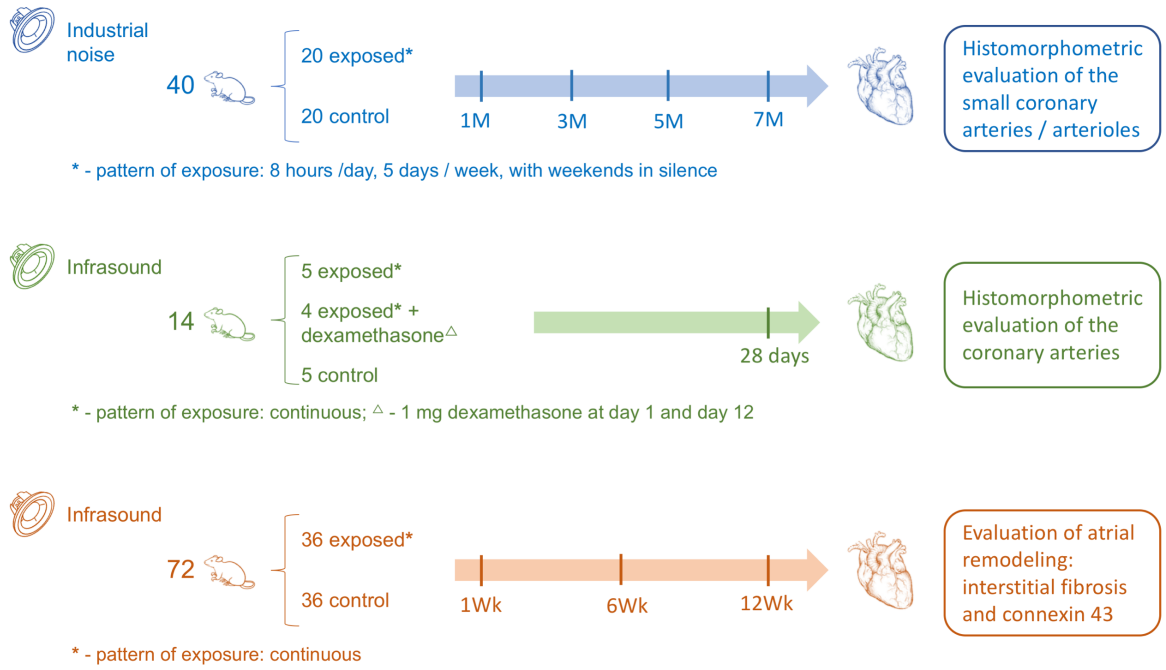

Figure 1. The three series of Wistar rats evaluated in this thesis. In blue: histomorphometric evaluation of the small coronary arteries in rats exposed to industrial noise; in green: the study of the morphological changes induced by IFS in the presence and absence of an anti-inflammatory agent; in orange: evaluation of atrial remodeling in rat hearts after exposure to high intensity IFS.

3. HISTOMORPHOMETRIC EVALUATION OF THE SMALL CORONARY ARTERIES IN RATS EXPOSED TO INDUSTRIAL NOISE

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HISTOMORPHOMETRIC EVALUATION OF THE SMALL CORONARY ARTERIES IN RATS EXPOSED TO INDUSTRIAL NOISE

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Abstract

Morphological changes induced by industrial noise (IN) have been experimentally observed in several organs. Histological observations of the coronary arteries showed prominent perivascular tissue and fibrosis among IN-exposed rats. The effects on the small arteries are unknown. Objective: To evaluate the histomorphometric changes induced by IN on rat heart small arteries. Methods: Twenty Wistar rats exposed to IN during a maximum period of seven months and 20 age-matched controls were studied. Hearts were transversely sectioned from ventricular apex to atria and a mid-ventricular fragment was selected for analysis. The histological images were obtained with an optical microscope using 400× magnifications. A total of 634 arterial vessels (298 IN-exposed and 336 controls) were selected. The mean lumen-to-vessel wall (L/W) and mean vessel wall-to-perivascular tissue (W/P) ratios were calculated using image J software. Results: There were no differences between exposed and control animals in their L/W ratios (p = 0.687) and time variations in this ratio were non-significant (p = 0.110). In contrast, exposed animals showed lower W/P ratios than control animals (p < 0.001), with significant time variations (p = 0.004). Conclusions: Industrial noise induced an increase in the perivascular tissue of rat small coronary arteries, with significant development of periarterial fibrosis.

Keywords: industrial noise, small coronary arteries, low-frequency noise

1. Introduction

Industrial noise (IN) is characterized by high intensity and a wide spectrum of wavelengths that includes low-frequency noise (LFN), this last characterized by large pressure amplitude ≥90 dB and low-frequency bands of ≤500 Hz [1]. Several morphological changes induced by IN and LFN have been experimentally observed in several tissues and organs [1-7].

We previously reported that coronary artery vessels showed prominent perivascular tissue and fibrotic development among IN-exposed rats [2] and also a significant fibrotic development in ventricular myocardium of rats exposed to LFN [3]. Considering the epidemiological evidence relating noise to ischemic heart disease and hypertension [8] and the effects of LFN on the extracellular matrix between the cardiomyocytes and around the cardiac vessels, which ultimately lead to myocardial stiffness and left ventricular disfunction and possibly to cardiac heart failure and arrhythmias [9, 10], additional studies were performed. These showed a reduction of cardiac connexin 43 and a significant increase of cardiac collagen I and III after LFN exposure [4, 5], reinforcing the hypothesis of an inducible morphological arrhythmogenic substrate.

The effects on the morphology of small arteries and arterioles in the rat heart are currently unknown, namely to what extent arteriolar wall thickening and perivascular fibrosis of the heart can be influenced by IN and LFN.

The aim of this study was to characterize the structural changes induced by IN on the rat heart small arteries.

2. Material and methods

Forty adult Wistar rats were studied. The animals were treated in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes (86/609/EEC) and with the Portuguese legislation for the same purpose (Decree-Law No. 197/96). All the animals were kept in cages, fed standard rat food, and had free access to water. Twenty animals (Group A) were exposed to IN for a period of one to seven months, in an occupationally simulated schedule (8 hours/day, 5 days/week, and weekends in silence). The remaining 20 rats were used as age-matched controls (Group B) and were kept in a silent environment. Each group was divided into four subgroups with five rats and sacrificed after 1, 3, 5 and 7 months.

The sound signal was emitted by an analog noise generator, amplified and frequency filtered. The noise level was the same as previously reported, characterized by a wide spectrum of frequencies but with an important component under 500 Hz [11].

The hearts were fixed in 10% buffered formalin, sectioned transversely from the ventricular apex to the atria and prepared for histological observation using hematoxylin eosin and chromotrope-aniline blue (CAB) staining. The mid-ventricular fragment from each heart was selected for the study. The histological images were acquired with an optical microscope using 400× magnification.

A total of 634 arterial vessels were selected (298 in group A and 336 in group B). Data were analyzed using the computer image analysis image J software (National Institutes of Health, Bethesda, MA, USA). The caliber of the arterial vessels, the thickness of the walls and the perivascular tissue dimension were measured and the mean lumen-to-vessel wall (L/W) and mean vessel wall-to-perivascular tissue (W/P) ratios were calculated.

Statistical Analysis

The morphometric data are presented as mean ± standard deviation. A two-way ANOVA model was applied in order to compare animals exposed to noise with non-exposed age-matched controls, in what concerns time variations (at months 1, 3, 5 and 7) in the L/W and W/P ratios assessed in the cardiac muscle, *in totum* and per anatomic region (right ventricle, septum, and left ventricle). The appropriateness of the ANOVA model is justified by the fact that the 8 subgroups defined by the factors levels are of equal size (n= 5), which warrants the robustness of the F ratio statistics under potential heterogeneous variances. A p value <0.05 was considered statistically significant.

3. Results

3.1. Data in *Totum*

3.1.1. Lumen-to-Vessel Wall Ratio

We found no differences in the lumen-to-vessel wall (L/W) ratio between exposed and non-exposed animals. The histological evaluation did not show the presence of inflammatory cells in the two groups or modifications in the lumen or in the vessel wall.

The results of the histomorphometric analysis are shown in Table 1. The mean L/W ratio was 0.5560 and 0.5619, respectively, in groups A and B.

Table 1. Lumen-to-vessel wall ratio descriptive statistics in each group and overall, at the different times.

Group	Exposure Time (Months)	Mean	Standard Deviation	*N*
IN exposed (group A)	1	0.5828933	0.03119288	5
	3	0.5579890	0.02053823	5
	5	0.5361110	0.04120586	5
	7	0.5471695	0.04270947	5
	Total	0.5560407	0.03675745	20
Control (group B)	1	0.5301545	0.04229613	5
	3	0.5820185	0.06370201	5
	5	0.5242906	0.06810608	5
	7	0.6111611	0.03569630	5
	Total	0.5619062	0.06211472	20

IN = industrial noise

The two-way ANOVA analysis of the data showed no differences between exposed and control animals in their L/W ratios (p = 0.687) and the time variations in this ratio were not significant (p = 0.110), as shown in Figure 1.

However, since a significant interaction between the independent variables was found in the model (p = 0.046), with an observed power of 64.9%, we performed multiple comparisons by means of planned contrasts at different times that showed that, at month 7, there were significant differences between groups for this ratio *in totum*, with significantly decreased values in exposed animals compared to controls (p = 0.034).

Regarding the differences between the groups in what concerns the time variations in this ratio, such differences were significant only for the changes observed from month 1 to 7 (p = 0.007), expressed as a 15.3% increase in the control group *versus* a 6.2% decrease in the exposed animals.

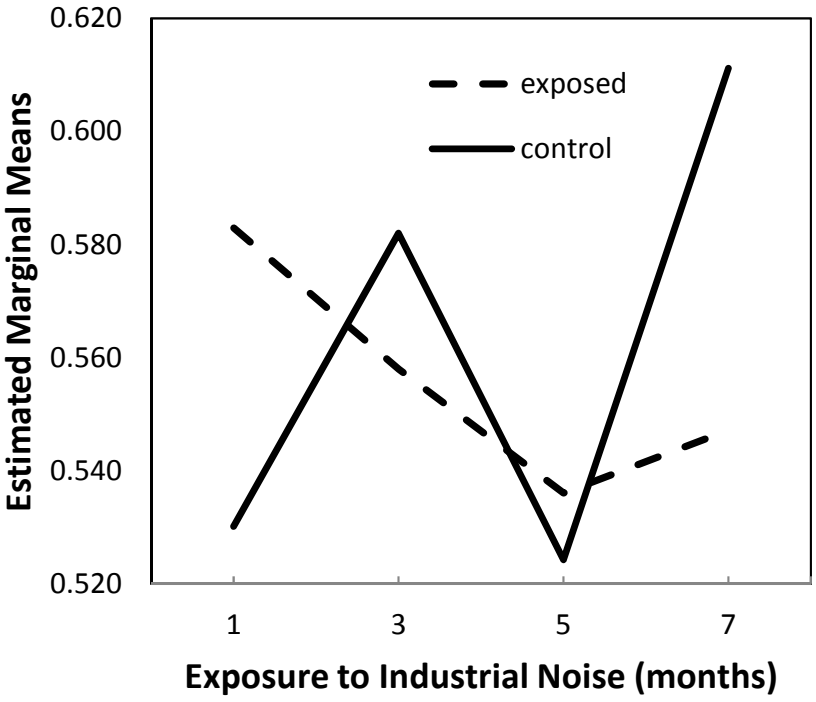

Figure 1. Estimated marginal means of lumen-to-vessel wall ratio.

3.1.2. Wall-to-Perivascular Tissue Ratio

By contrast, the perivascular tissue was more prominent and seemed to show fibrosis in IN-exposed rats. Sections of the arteries are shown in Figure 2.

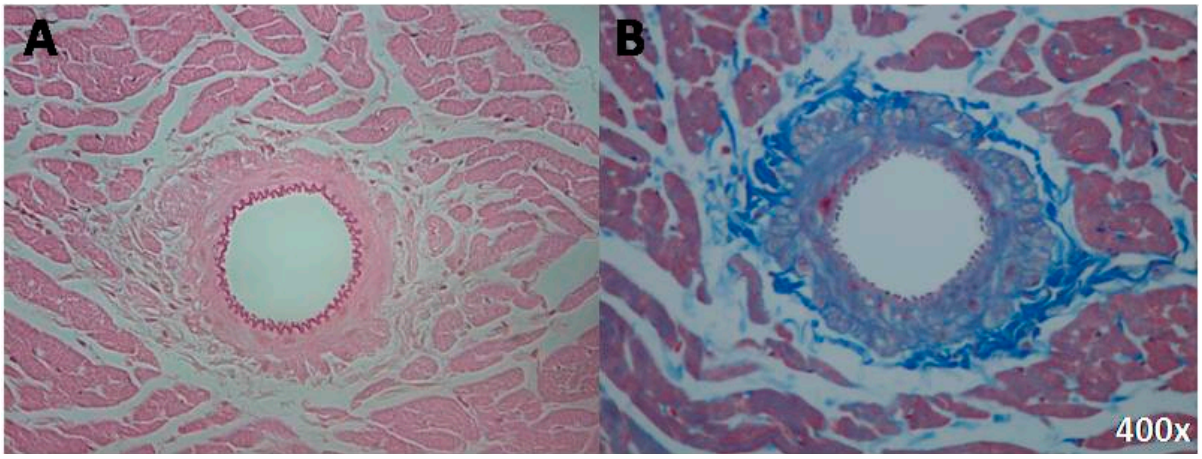

Figure 2. (A) Small coronary artery with a prominent perivascular tissue (IN group) (Hematoxylin-Eosin, 400×); (B) Small coronary artery with a prominent perivascular tissue, in the same group (Chromotrope-Aniline blue, 400×).

The mean wall-to-perivascular tissue (W/P) ratio was 0.4209 and 0.6373, respectively, in groups A and B (Table 2).

Table 2. Wall-to-perivascular tissue ratio descriptive statistics in each group and overall, at the different times.

Group	Exposure Time (Months)	Mean	Standard Deviation	*N*
IN exposed (group A)	1	0.4322535	0.03050318	5
	3	0.3986722	0.02655070	5
	5	0.4234890	0.05262512	5
	7	0.4292097	0.04201934	5
	Total	0.4209061	0.03850858	20
Control (group B)	1	0.5849600	0.04896260	5
	3	0.6778928	0.01880381	5
	5	0.5745753	0.07750708	5
	7	0.7117764	0.01747083	5
	Total	0.6373011	0.07454984	20

IN = industrial noise

The results of the two-way ANOVA showed that there were significant effects on W/P ratio due to exposure ($p < 0.001$) and time variations ($p = 0.004$), which, however, are secondary, since a significant interaction between exposure and time exists ($p = 0.001$), as shown in Figure 3.

It is important to note that these effects do not seem to be due to chance, as the observed power is in excess of 90.7%.

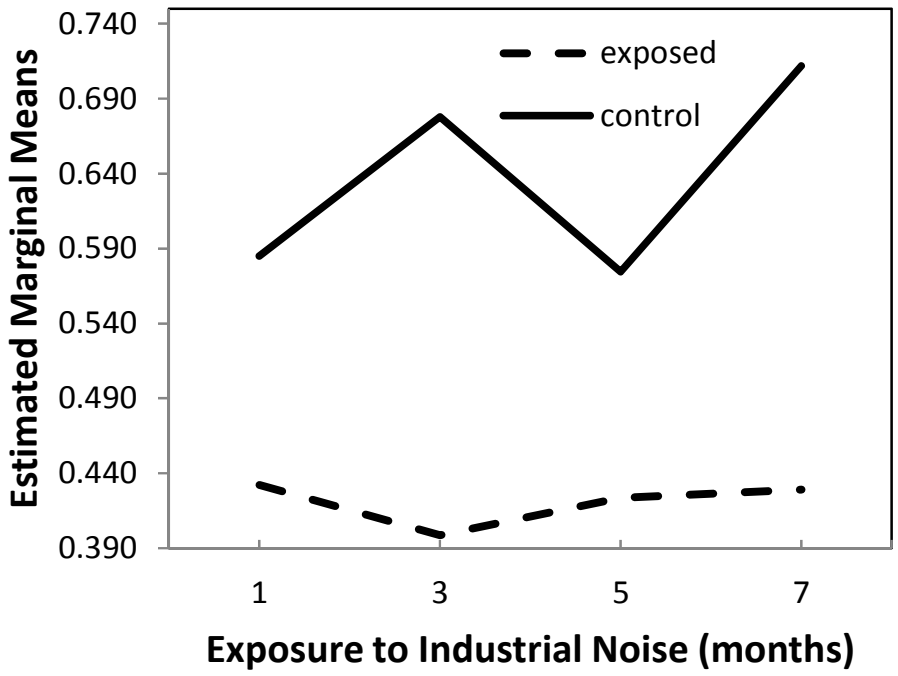

Figure 3. Estimated marginal means of wall-to-perivascular tissue ratio.

In view of this significant interaction, planned contrasts were applied in the *post hoc* comparisons, which allow the following conclusions:

(1) At months 1, 3, 5 and 7, exposed animals have significantly lower W/P ratio than control animals ($p < 0.001$);

(2) From month 1 to 3, there is a 7.8% decrease in W/P ratio in exposed animals which differs significantly ($p = 0.003$) from the 15.9% increase in the same ratio observed in the control animals;

(3) From month 3 to 5, there is a 6.0% increase in W/P ratio in exposed animals, which differs significantly ($p = 0.002$) from the 15.2% decrease observed in the control animals;

(4) From month 5 to 7, there is an increase of 1.4% in W/P ratio in exposed animals, which is significantly lower ($p = 0.002$) than that of 23.8% observed in control animals.

3.2. Data Per Anatomic Region

3.2.1. Lumen-to-Vessel Wall Ratio

The analysis of the data has shown that there were no significant effects of exposure to noise, duration of exposure and interaction between such factors on the L/W ratio in any of the anatomic regions considered: right ventricle (RV), septum and left ventricle (LV).

3.2.2. Wall-to-Perivascular Tissue Ratio

Right Ventricle

Significantly decreased W/P ratios were observed in the RV of exposed animals comparatively to controls ($p > 0.001$). No effects of duration ($p = 0.111$) or interaction between exposure and duration ($p = 0.208$) were observed in the same anatomic region.

Septum

The Two-way ANOVA approach to the data shows that there were significant effects on the W/P ratio in septum due to exposure ($p < 0.001$) and duration ($p = 0.006$), which were secondary, because of the significant interaction between exposure and duration ($p = 0.002$). It is important to note that these effects did not seem to be due to chance, as the observed power was in excess of 87.7%.

In view of the significant interaction, planned contrasts were applied in the *post hoc* comparisons, with the following conclusions:

(1) Exposed animals had significantly lower W/P ratio in septum than control animals, at all times ($p \leq 0.006$);

(2) From month 1 to 3, there was a 13.8% decrease in W/P ratio in exposed animals which differed significantly ($p = 0.003$) from the 18.8% increase in the same ratio observed in the control animals;

(3) From month 3 to 5, there was a 13.2% increase in W/P ratio in exposed animals which differed significantly ($p = 0.004$) from the 16.7% decrease observed in the control animals;

(4) From month 5 to 7, there was an increase of 3.1% in W/P ratio in exposed animals which was significantly lower ($p = 0.006$) than that of 30.6% observed in control animals.

Left Ventricle

The Two-way ANOVA showed that there were significant effects on the W/P ratio in the LV due to exposure ($p < 0.001$) but not to duration ($p = 0.282$), and a significant interaction between exposure and duration was found ($p < 0.001$). Again, these effects did not seem to be due to chance, as the observed power was in excess of 98.3%.

Planned contrasts were applied in the *post hoc* comparisons, with the following conclusions:

(1) Exposed animals had significantly lower W/P ratio in LV than control animals, at all times ($p < 0.001$);

(2) From month 1 to 3, there was a 9.9% decrease in W/P ratio in exposed animals which differed significantly ($p = 0.010$) from the 12.9% increase in the same ratio observed in the control animals;

(3) From month 3 to 5, there was a 14.0% increase in W/P ratio in exposed animals which differed significantly ($p = 0.006$) from the 10.8% decrease observed in the control animals;

(4) From month 5 to 7, there was a decrease of 12.3% in W/P ratio in exposed animals which was significantly different ($p = 0.006$) from the increase of 20.3% observed in control animals.

4. Discussion

A number of animal studies found modifications in several tissues induced by low-frequency noise, characterized by abnormal deposition of collagen in the extracellular matrices [1-7].

In the present study we found an increase in the perivascular tissue around the small coronary arteries in rats exposed to IN. This is in agreement with the previous findings of our group, concerning the histomorphometric evaluation of the coronary arterial vessels in rats exposed to industrial noise [3]. In both studies we found the development of perivascular fibrosis in the absence of inflammatory cells and in the absence of obstructive coronary artery disease.

There were significant differences between exposed rats and controls concerning the mean vessel wall-to-perivascular tissue ratio, higher among the control group ($p < 0.001$). The effects of exposure time were observed in the whole population of rats and seemed to be independent of exposure to IN. The perivascular tissue was more exuberant at three months of IN exposure, with a gradual reduction observed until seven months. It is important to note that, despite this

gradual reduction, it remained increased among the IN-exposed rats compared to control at all times.

The analysis of data per anatomic region showed no particular differences between the right ventricle, septum and left ventricle, confirming that the influence of industrial noise over the heart is global.

Regarding the results concerning the ratio lumen-to-vessel wall between exposed and non-exposed animals, no significant differences were found until seven months of exposure to IN. At this point, an increased wall thickness of intramyocardial small coronary arteries was observed in exposed rats, as compared to controls ($p = 0.034$). Previous studies performed on large vessels found the same type of modifications in the vessel wall [6]. In contrast, such differences were not observed in the coronary arterial vessels [3]. In this case, we speculated that a lower susceptibility of coronary arterial vessels to IN damage may occur, as suggested by the absence of the internal elastic lamina disruption and the fact that no proliferation of smooth muscle cells was observed in the intima up to seven months of exposure. Taking into consideration our recent results, we may further speculate that industrial noise effects on wall thickening require longer times of exposure and that small coronary arteries are the first to be affected.

A previous review of epidemiological studies concerning environmental noise exposure (including road and aircraft noise sources) and cardiovascular risk reported increasing evidence relating noise and hypertension and ischemic heart disease [8], making pertinent to investigate the effects of IN on the heart.

Thus far, we documented that coronary artery vessels showed prominent perivascular tissue and fibrotic development among IN-exposed rats [3] and also a significant fibrotic development in ventricular myocardium of rats exposed to LFN [2]. Considering that these structural changes ultimately lead to myocardial stiffness and left ventricular disfunction and possibly to cardiac heart failure and arrhythmias [9, 10], additional studies were performed. These showed a reduction of cardiac connexin 43 and a significant increase of cardiac collagen I and III after LFN exposure [4, 5], reinforcing the hypothesis of an inducible morphological arrhythmogenic substrate. The present study allowed the documentation of structural changes induced by industrial noise on the rat heart small arteries, suggesting a general influence of industrial noise over the heart.

It remains to understand the fibrotic proliferation mechanism behind the IN. A strong possibility, taking into consideration the dynamic interactions between fibroblasts and the extracellular matrix [11], could be the occurrence of an abnormal biological fibroblastic response induced

by IN through a mecanotransduction process [12]. In addition, we could speculate that IN can induce fibrosis through the loss of regulation between profibrotic and antifibrotic molecules, carried out by mechanical and neurohumoral factors [13].

The changes in the structure of coronary resistance vessels, with quantitative characterization of small coronary arteries and arterioles in the myocardium, have been extensively studied under several experimental conditions and were already extended to humans. Cardiac hypertrophy in hypertension, with an increase in left ventricular mass, is characterized by increased wall thickness in arterioles and small arteries, increased lumen to wall ratio, and decreased number of capillary profiles per arteriole in cross section [14]. Volume overload-induced cardiac hypertrophy is characterized by normal coronary reserve and maximal flow and there is evidence that both arteriolar and capillary growth is proportional to the magnitude of hypertrophy [15]. Pathologic findings have described diabetic cardiomyopathy as a microvascular disease and previous studies have demonstrated that structural changes of coronary microvessels in diabetes include thickening of the vascular wall, perivascular fibrosis, capillary aneurysms, and decrease in capillary density [16, 17]. Several structural abnormalities of the heart are present in chronic renal failure, including arteriolar thickening [18], reduced capillary density [19], and interstitial fibrosis [20]. These findings contribute to myocardial ischemia, left ventricular wall stiffness, diastolic dysfunction, and arrhythmogenicity in patients with renal failure [21].

It will be our challenge in the future to understand the clinical impact on cardiac diseases among humans exposed to industrial and low-frequency noise.

We are aware of several limitations of the study. We admit that interpretation of the results must be done cautiously because, for logistical reasons, the number of animals per group was limited. Additionally, small coronary arteries in biopsy samples were partially crushed during the procedure, which might have affected the quantitative analyses of lumen area and perivascular tissue. Furthermore, we acknowledge that at the present time there is not a well-defined morphological cardiac model induced by industrial noise. Thus far, we have limited our observations to the structural modifications induced by industrial noise or by low-frequency noise in the myocardium of rat heart and can only extrapolate these observations to humans, with all the existing limitations. The experimental conditions tried to simulate the schedule of industrial plant workers, characterized by 8 hours/day, 5 days/week of exposure to industrial noise. Once again, we reinforce the need of clinical investigations concerning the effects of industrial and low-frequency noise on the heart.

5. Conclusions

In conclusion, industrial noise induced an increase in the perivascular tissue of rat small coronary arteries, with significant development of periarterial fibrosis.

Author contributions

Ana Lousinha conceived and designed the experiments, performed the histomorphometric evaluation, analyzed the data and wrote the article; Eduardo Antunes conceived and designed the experiments, performed the histomorphometric evaluation and participated in the revision of manuscript content; Gonçalo Borrecho performed the experiments and participated in the histomorphometric evaluation; Maria João Oliveira conceived the experiments; José Brito performed the statistical analysis; José Martins dos Santos participated in the revision of manuscript content. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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4. INFRASOUND INDUCES CORONARY PERIVASCULAR FIBROSIS IN RATS

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Infrasound induces coronary perivascular fibrosis in rats

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Abstract:

Background: Chronic exposure to industrial noise is known to affect biological systems, namely by inducing fibrosis in the absence of inflammatory cells. In rat hearts exposed to this environmental hazard, we have previously found myocardial and perivascular fibrosis. The acoustic spectrum of industrial environments is particularly rich in high-intensity infrasound (IFS) (<20 Hz), whose effects on the heart are unknown. We evaluated the morphological changes induced by IFS in rat coronaries in the presence and absence of dexamethasone. **Methods:** Adult Wistar rats were divided into three groups: group A (GA)-IFS (<20Hz, 120dB)-exposed rats for 28 days treated with dexamethasone; group B (GB)-IFS-exposed rats; group C (GC)-age-matched controls. The midventricle was prepared for observation with an optical microscope using 100× magnification. Thirty-one arterial vessels were selected (GA 8, GB 10, GC 13). The vessel caliber, thickness of the wall, and perivascular dimensions were quantified using image J software. Mann-Whitney and Kruskal-Wallis tests were used to compare the groups for lumen-to-vessel wall (L/W) and vessel wall-to-perivascular tissue (W/P) ratios. **Results:** IFS-exposed rats exhibited a prominent perivascular tissue. The median L/W and median W/P ratios were 0.54 and 0.48, 0.66 and 0.49, and 0.71 and 0.68, respectively, in GA, GB, and GC. The W/P ratio was significantly higher in GC compared with IFS-exposed animals ($p=0.001$). The difference was significant between GC and GB ($p=0.008$) but not between GC and GA. **Conclusion:** IFS induces coronary perivascular fibrosis that differs under treatment with corticosteroid.

Keywords: Infrasound, low frequency noise, coronary arteries, fibrosis, inflammation.

1. Introduction

Noise represents a major environmental factor and is among the stressors with the highest impact on public health [1]. Noise and sound are physically the same, but the reaction to perception varies between people, depending on the cognitive environment in which detection takes place and ultimately leads to a definition of noise as an undesired sound [2, 3]. Low-frequency noise (LFN) and infrasound (IFS) are conventionally defined as sound below 200 and 20 Hz, respectively. The lower limit of the audio frequency range of human hearing is usually given as 16 or 20 Hz, but humans can perceive infrasound if the sound pressure level (dB) is sufficiently high [4]. In the range of IFS, comparative studies have shown that the auditory sensitivity of different species can vary widely. For instance, rats have poorer infrasonic hearing than humans, considering different sound pressure levels [5], but high-intensity (110 dB) IFS vibrations on experimental rats can be perceived, as they elicit active avoidance reactions [6]. Beside its auditory health effects, noise can cause non-auditory effects - such as annoyance, sleep disturbance, and psychological stress - that experimental and epidemiological evidence links to cardiovascular disease, including ischemic heart disease, heart failure, arterial hypertension, arrhythmia and stroke [7-12].

In recent years, scientists have directed their attention towards the relatively understudied noise range of below 200 Hz. LFN and IFS are present everywhere, from natural occurrences to industrial installations and low-speed machinery. The characteristics of strong penetration and less attenuation in long distance propagation have been proposed to explain several adverse biological effects in experimental and epidemiological studies [13]. Low-frequency sounds have higher energy than the sounds at mid and higher frequencies and cannot be correctly evaluated using the conventional A-filters, which are most often used in environmental studies [14]. It is also possible that there are subtle effects of LFN on the body that we do not yet understand. High sound pressure levels (≥ 90dB) of LFN can induce resonance responses in body cavities [13]. The overall range of human body resonant frequencies was found to be from 2 to 16 Hz [15], which is nearly the exact range of IFS. It may be assumed that animals also possess inherent specific sound frequencies in certain tissues and organs [16], and for that reason, it is important to document, using animal models, the morphological and biological effects induced by a wide spectrum of wavelengths, from industrial to LFN and IFS.

The cardiovascular system of rodents is sensitive to LFN [17-19]. We previously documented the development of perivascular fibrosis around the coronary arteries (from small to large caliber) of rats exposed to industrial noise [20, 21]. We also found a significant fibrotic

development in ventricular myocardium among rats submitted to LFN [22, 23]. These morphological changes were found in the absence of inflammatory cells, which could suggest a non-inflammatory process. However, the fibrotic proliferation mechanism remains unclear.
The effects of IFS on the coronary artery morphology under the influence of an anti-inflammatory agent are unknown. In order to fill this gap, we sought to evaluate the morphological changes induced by IFS in rat coronary arteries in the presence and absence of dexamethasone.

2. Material and Methods

Fourteen adult female Wistar rats 10 months old were used in this study. They were purchased from a Spanish breeder (Charles River Laboratories España, S.A., Spain). All the handling and care of the experimental animals were performed by authorized researchers (accredited by the Federation of European Laboratory Animal Science Associations, Category C) and were done in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes (2010/63/EU) and with the Portuguese legislation for the same purpose (Decree-Law No. 197/96). The rats were housed in 42×27×16-cm polypropylene cages with a steel lid and had unrestricted access to food (commercial chow) and water. The same standard house conditions were used throughout the experiment for all the animals, and they involved keeping a maximum of two rats in a single cage.

In the beginning of the study, the 14 rats were randomly distributed into three groups. Nine of the rats were continuously exposed to high-intensity and very LFN (2–20Hz / Lp=114dB) during a period of 28 days. In four of the noise-treated rats, two tablets of dexamethasone 0.5 mg (Decadron 0.5 mg, Medinfar) were introduced subcutaneously in the dorsal region at two time points of the noise exposure, day one and day 12, and these were designated as group A, while the dexamethasone-free rats were included in group B. The remaining five rats were used as age-matched controls (group C) and sacrificed when all of the rats reached 11 months of age.

2.1. Short description of electroacoustic experiment

With the objective of creating a strong subsonic acoustic field in the vivarium chamber, a slightly trapezoidal room with 23.7m3 (3.55×3.31×2.02, average length×width×height, respectively, in meters), a pseudo-random waveform in the 2-Hz to 20-Hz decade band was

designed with Matlab based on a bandpass-filtered 30-s maximum length sequence segment. The waveform was used to excite an array of two infinite baffles mounted 18-in. 300-W-rated magnetodynamic subwoofers, by means of a 2×600-W heavy-duty quasi-dc voltage output audio power amplifier. Subsequently, with the aim of exploiting as much as possible the available subwoofers dynamic range at this frequency range with an acceptable amplitude distortion, the waveform was iteratively nonlinearly treated with moderate compression-expansion and further filtering (in order to reduce the crest factor to approximately 2.0 times). The total sound pressure level and the spectral characteristics of the resulting acoustic pressure waveform were monitored, and the results were an average sound pressure level of 120 dB with a tolerance of ±3 dB in the 30-s time window. As to the spectral boundedness of the produced sound field, the result was 80 dB total out-of-band average sound pressure level (−40dB lower).

2.2. Light microscopy

All rats were sacrificed by an intravenous injection of 0.6 mL of a 5:4 mixture containing ketamine (Imalgene 1000, Bayer, Portugal) and xylazine (Rompun, Bayer, Portugal). The vascular system was perfused with a saline solution followed by paraformaldehyde fixation. The heart was excised, sectioned transversely from the ventricular apex to the atria, and routinely processed for light microscopy. The midventricular fragment from each heart was selected for the study. Five-micrometer paraffin-embedded slices of the tissue samples were made and dyed according to Sirius red techniques. The histological images were acquired with an optical microscope using 100× magnification.

2.3. Histomorphometric data

Thirty-one arterial vessels were selected (8 in GA, 10 in GB, and 13 in GC) (Figure 1). At least one vessel from each rat was included. The researchers, including data collectors and data analysts, were blinded to which group the animals belonged to. Data were analyzed using the image J software (National Institutes of Health, Bethesda, MD, USA). The caliber of the arterial vessels, the thickness of the walls, and the perivascular tissue dimension were measured, and for each rat, the mean lumen-to-vessel wall (L/W) and mean vessel wall-to-perivascular tissue (W/P) ratios were calculated (Figure 2). (See Table 1)

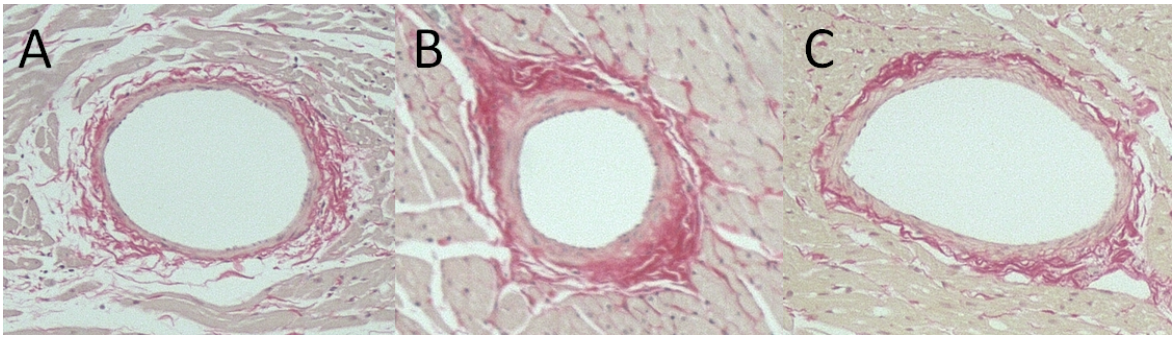

Figure 1. Coronary artery vessels in fragments taken from the left mid-ventricle from (A) group A, infrasound exposed dexamethasone-treated rats (B) group B, infrasound-exposed rats and (C) group control. Note de prominent perivascular tissue in infrasound-exposed animals [Sirius Red, 100x].

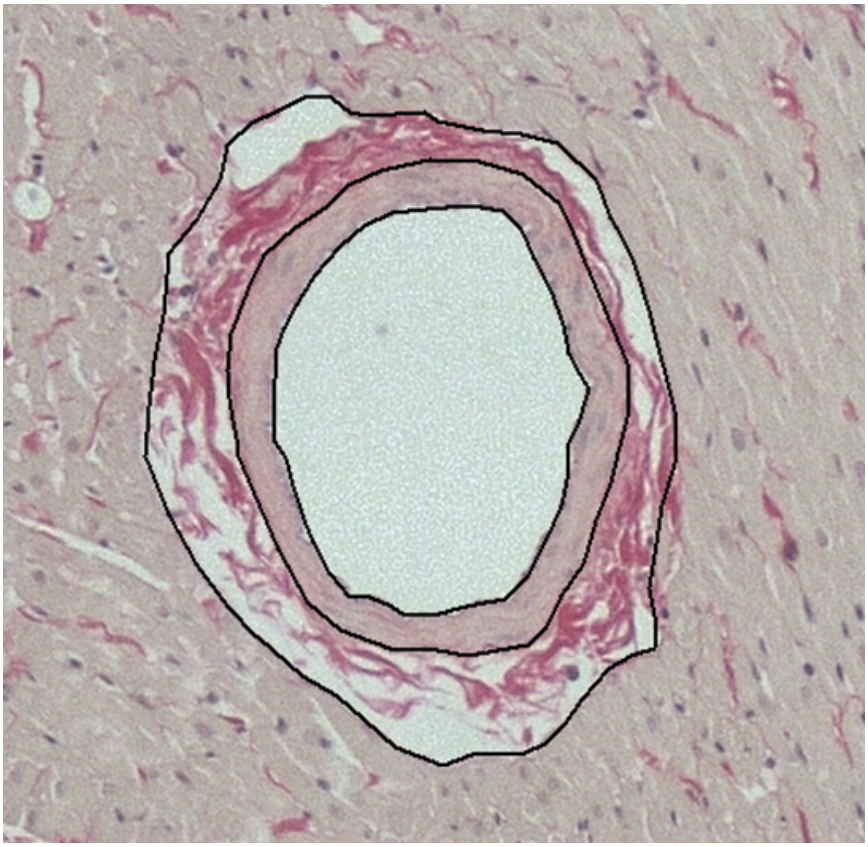

Figure 2. Example of a coronary artery in a fragment taken from the left mid-ventricle of an infrasound exposed rat [Sirius Red, 100x]. The black lines represent the measurements performed using *Image j* software and correspond to vessel calibre, thickness of the wall and perivascular dimension. These were used to calculate the ratio lumen-to-vessel wall (L/W) and ratio vessel wall-to-perivascular tissue (W/P).

2.4. Statistical analysis

Mann–Whitney test has been applied in the comparison of IFS-exposed animals (including animals treated with dexamethasone and nontreated animals) and a control group for two parameters: L/W and W/P ratios. Kruskal–Wallis and Mann–Whitney tests were used in the comparison of the three groups for the same parameters. A p value < 0.05 was considered statistically significant.

3. Results

Table 1. Median (interquartile range) of the two measured outcomes in the three groups

	Ratio L/W Median (interquartile range)	Ratio W/P Median (interquartile range)
Group A	0.54 (0.17)	0.48 (0.15)
Group B	0.66 (0.09)	0.49 (0.08)
Group C	0.71 (0.10)	0.68 (0.08)

L/W - lumen-to-vessel wall; W/P - vessel wall-to-perivascular tissue

3.1. IFS-exposed animals vs. control animals

The Mann–Whitney test has been used to compare the two groups for L/W ratio and W/P ratio variables, with the Bonferroni correction $\alpha^* = 0.05/2=0.025$. The analysis shows that the W/P ratio is significantly lower in the IFS-exposed group ($p=0.001$). In contrast, the L/W ratio did not differ between the two groups ($p=0.060$). It should be mentioned that the extreme observation for W/P ratio values in the control group does not influence these conclusions, as differences between the groups were still detected by the Mann–Whitney test after removal of that observation ($p=0.003$), as expected in view of the robustness of this nonparametric test against such extreme values (figure 3).

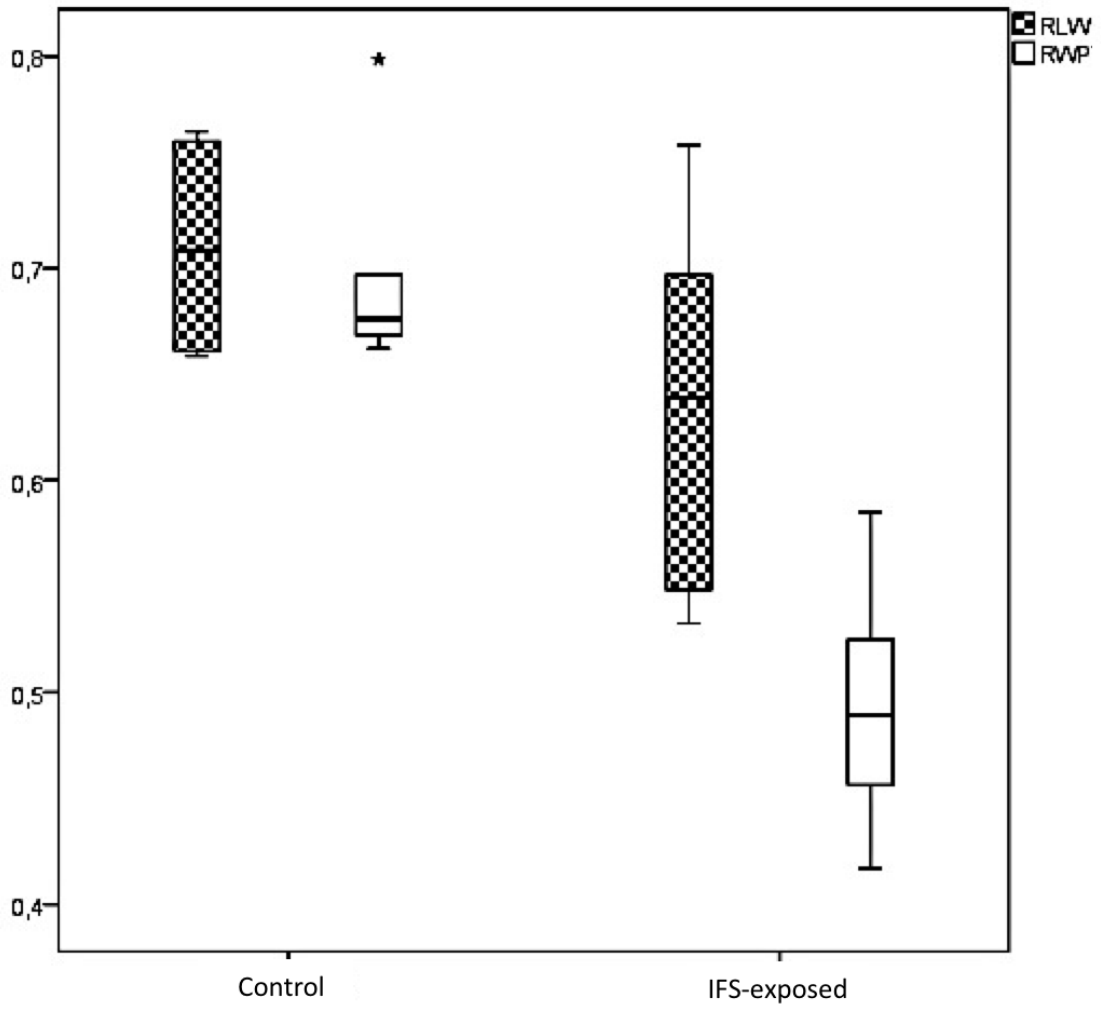

Figure 3. Ratio lumen-to-vessel wall and ratio vessel wall-to-perivascular tissue in IFS-exposed and control animals. The ratio W/P was significantly reduced in IFS-exposed animals (p = 0.001). RLW – ratio lumen-to-vessel wall, RWP – ratio vessel wall-to-perivascular tissue.

3.2. Comparison between IFS-exposed dexamethasone-treated animals, IFS-exposed animals, and control animals

In the comparison between the three groups, the Kruskal–Wallis test has been applied with the same Bonferroni correction to the significance level, $\alpha^* = 0.025$. The analysis has shown that there are differences between the groups for W/P ratio (p=0.011), but not for L/W ratio (p=0.104). Post hoc comparisons between the groups were conducted for W/P ratio, using the Mann–Whitney test, at the 0.025/3 = 0.0083 significance level to control for inflation of type 1 error. In this case, differences were detected between control and IFS-exposed animals not

treated with dexamethasone (p=0.008). It should be mentioned that the extreme observation of W/P ratio values does not seem to influence the main conclusion of the Kruskal–Wallis test, as expressed by a significance of 0.021 of the test result after removal of that observation, but it does change the conclusions of the Mann-Whitney test in the comparison between groups B and C, which is now nonsignificant (p=0.021) under the Bonferroni correction (α* = 0.0083) (figure 4).

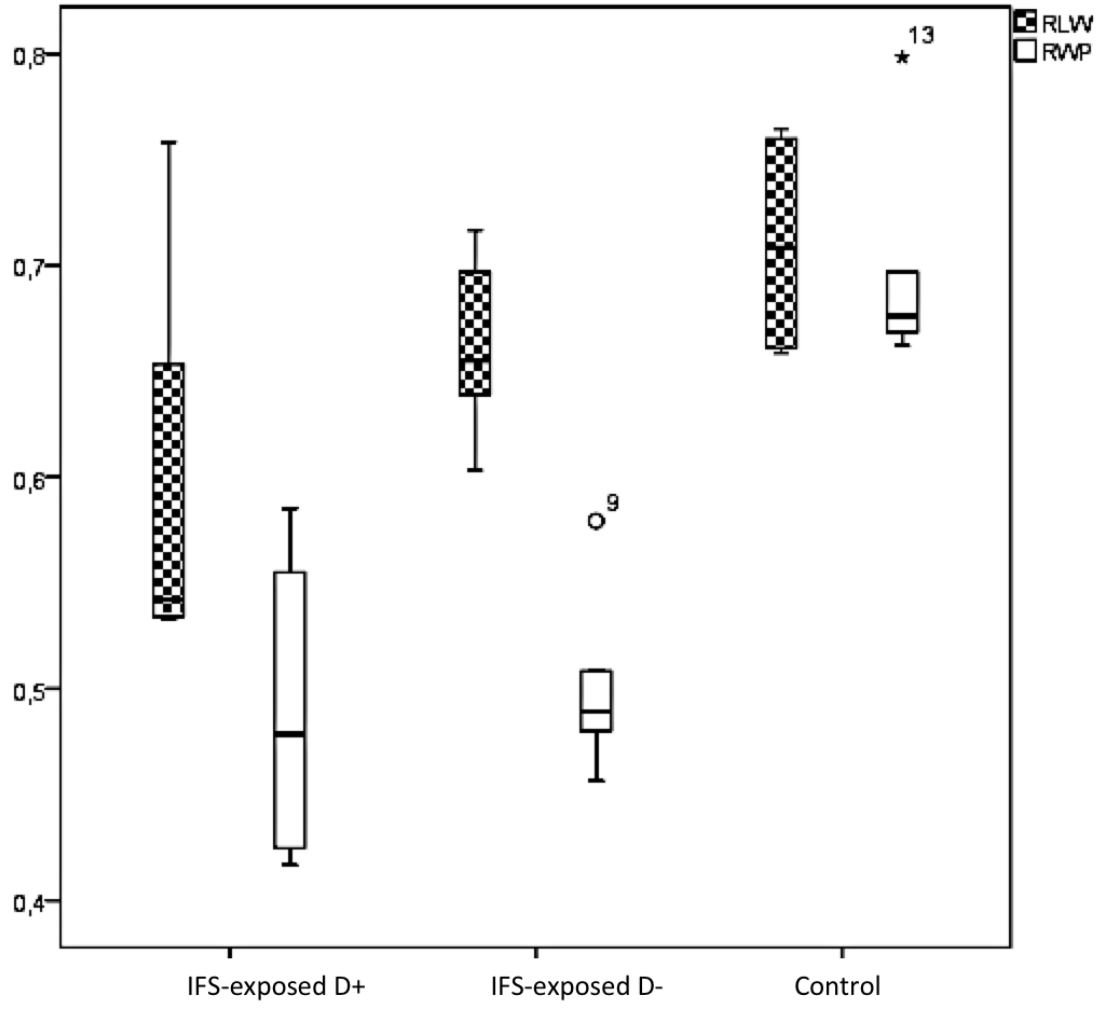

Figure 4. Ratio lumen-to-vessel wall and ratio vessel wall-to-perivascular tissue in infrasound-exposed dexamethasone-treated rats (group A), infrasound-exposed rats (group B) and group control (group C). For ratio W/P, there are differences between the groups (p = 0.011) and between groups B and C (p = 0.008), but not between groups A and C. RLW – ratio lumen-to-vessel wall, RWP – ratio vessel wall-to-perivascular tissue, D+ and D- - dexamethasone-treated and not treated, respectively.

4. Discussion

The present study evaluated the coronary morphological changes in rat heart induced by pure IFS, created in a laboratory controlled electroacoustic experiment, and is the first study assessing the possible influence of an anti-inflammatory agent on these changes.

In this investigation, we found an increase in the perivascular tissue around the coronaries in rats exposed to IFS. There were significant differences between IFS-exposed rats and controls concerning the mean ratio W/P, higher among the control group ($p < 0.001$). But such differences did not reach statistical significance in the comparison between the animals treated with dexamethasone and the control group, pointing to a possible influence of this potent anti-inflammatory agent.

Previous work from our group, in Wistar rats, investigated the histomorphometric changes in the large and small coronary arteries induced by high intensity industrial noise within a wide spectrum of wavelengths that included LFN, this last characterized by large sound pressure amplitude $\geq$ 90dB and low frequency bands of $\leq$ 500Hz [20, 21]. The exposure time ranged from 1 to 7 months. In both studies, we found the development of perivascular fibrosis in the absence of inflammatory cells, regardless of exposure time. In another study, we have documented a significant fibrotic development in ventricular myocardium of rats exposed to LFN during a period of 3 months [22]. These investigations confirmed the abnormal proliferation of connective tissue as the main morphological change induced by LFN.

With increasing urbanization, noise is rising as one of the most important environmental risk factors in modern societies. The importance of the characteristics of the noise stimulus, such as frequency content, intensity, mean and peak dB level, pattern and exposure time, is not well understood. In the quantitative risk assessment of environmental noise, the World Health Organization (WHO) Regional Office for Europe is concerned with sound pressure level limits, not frequencies [1]. Nonetheless, WHO also acknowledges the special place of LFN as an environmental problem, recognizing that the evidence is sufficiently strong to warrant immediate concern.

Sources of LFN include natural occurrences, industrial installations and low-speed machinery, ranging from very low-frequency atmospheric fluctuations up to lower audio frequencies. Due to the characteristics of strong penetration and less attenuation in long distance propagation, it has been implicated in several adverse biological effects in experimental and epidemiological studies [13].

One effect of high pressure levels of LFN is excitation of body vibrations [13, 19, 24]. At high sound levels, typically above 80dB, the occurrence of resonance responses in body cavities was described [24]. The overall range of human body resonant frequencies was found to be from 2 to 16 Hz [15], which is almost the exact range of infrasound. The displacement between the organ and the skeletal structure places biodynamic strain on the body tissue involved and it is known to reach its maximum under exposure to vibration close to the body's resonant frequency. Despite the practical impossibility of stimulating the natural frequency of one organ alone without exciting the whole-body resonances, measurements of vibration transmissibility from the point of excitation to a specific organ reveals frequencies of maximum transmissibility that can be attributed to the resonance of the organ. Considering that animals also possess inherent specific sound frequencies in certain tissues and organs [16], it is important to assess the morphological and biological effects induced by noise with different wavelengths in distinct animal models. So far, we have focused our investigation on the effects of large pressure amplitude noise within a wide spectrum of wavelengths, from the industrial to LFN and IFS, and with different exposure times, from 1 to several months [20-23]. The common finding was an abnormal deposition of collagen in the extracellular matrix (ECM), regardless of the characteristics of the noise stimulus other than pressure amplitude.

Interest in the potential adverse health effects of IFS has increased over time. High level IFS below 20Hz was historically thought to be of much less significance than LFN in the 20-200Hz range at the same pressure level [25]. Research on the impact of IFS on the environment established that for levels above 120dB it is dangerous to the human body [13].

Infrasound exposure studies in laboratory animals are scarce and report adverse effects in the ear and auditory system [26], brain and central nervous system [27, 28], liver [29, 30] and lung [31]. Specifically, the cardiovascular system is sensitive to IFS, as shown by the first studies conducted more than 25 years ago. In these studies, rats were exposed to infrasound (4, 8 and/or 16Hz at 90 to 145dB) for up to 45 days that ultimately led to myocardial ischemia and morphofunctional changes in the myocardium cells [32-34]. More recently, Pei *et al* reported IFS-induced hemodynamics, cardiac ultrastructure damage and cardiac cell apoptosis in the rat myocardium [35, 36]. The same group found that IFS dysregulates the L-type calcium currents in rat ventricular myocytes [16], and also that acute exposure to IFS induces oxidative damage of cardiomyocytes that affects a series of oxidative damage-related proteins and genes, suggesting a complex signalling network that is evoked by this stressor [37].

There is no agreement about the biological activity of LFN and IFS and the possible underlying mechanisms. The biological effects of noise on living bodies may not be the same due to

different parameters such as biological species, frequency, level of sound pressure or time of exposure. Over the last years, an increased focus from investigators towards the elucidation of these questions has been observed. Increased release of stress hormones, activation of sympathetic nervous system, increased reactive oxygen species production, endothelial dysfunction, peripheral vasoconstriction, increased peripheral vascular resistance and increased blood viscosity are among the proposed mechanisms elicited by acute or chronic noise stress leading to detrimental outcomes on the cardiovascular system [7, 9, 38]. Following this line of investigation, Said and El-Gohary studied the effect of noise in the 80-100dB range on heart rate and mean systemic arterial blood pressure in adult male albino rats and explored possible underlying mechanisms [39]. They concluded that noise stress has many adverse effects on cardiovascular system through increasing plasma levels of stress hormones, oxidative stress and endothelial dysfunction.

Until recently, it was presumed that LFN required greater sound pressure in order to elicit toxicological effects on humans and animals. High sound pressure levels can be harmful to the cochlea and cause hearing loss, raising the question of other noise effects being secondary, at least partially, to direct auditory damage. Since animal models in previous studies employed mainly high dBA levels (> 100-120dBA), some investigators started exploring the effects of low decibel noise. Jin *et al* [17] used isolated and cultured cardiac fibroblasts from rats to study the effects of low decibel IFS. They reported that noise below 90 dB at 4-20Hz inhibits angiotensin II-stimulated cardiac fibroblasts by reactivating miR-29a targeting the TGF-β/Smad3 pathway, possibly eliciting cardiac protective effects. Münzel *et al* [18] developed a novel noise exposure model in mice with lower peak sound levels (<85dBA), lower mean sound pressure levels (72dBA) and shorter exposure times (1-4 days), thought to cause mainly non-auditory effects to animals such as stress reactions. Exposure to noise resulted in elevated blood pressure and heart rate, was associated with detrimental changes in vascular endothelial function, vascular production of reactive oxygen species, and increased blood stress hormones and biomarkers of inflammation. Notably, they describe an invasion of the vasculature with inflammatory cells. The same group demonstrated that nighttime aircraft noise in healthy volunteers causes endothelial dysfunction, which was partially corrected by the acute administration of vitamin C, pointing to increased oxidative stress as a key mechanism [40].

There is currently limited data on the hypothetical noise-induced pathway involving inflammation [11]. In humans, sleep disturbance is associated with a pro-inflammatory state [41]. As previously mentioned, the common finding in the noise experiments conducted by our group was the perivascular and myocardial fibrotic development in the absence of

inflammatory cells [20-23]. In the present study, we included a group of IFS-exposed animals treated with dexamethasone, a synthetic glucocorticoid member with immunosuppressive potency of about 20-30 times that of hydrocortisone and 4-5 times of prednisone [42, 43]. Subcutaneous application of dexamethasone, in contrast to intraperitoneal, is highly effective in inhibiting inflammation in mouse models, even at low doses [44]. Interestingly, we found differences in the comparison of control group with IFS-exposed animals with and without dexamethasone treatment, as the treated animals did not show significant differences when compared to controls. This is the first time that such differences are documented, and despite the absence of inflammatory cells previously described by our group we have to consider a potential underlying inflammatory mechanism.

The mechanism behind the fibrotic proliferation induced by noise in rat heart is not yet understood. In general, the differentiation of cardiac fibroblasts into more active myofibroblasts is the hallmark of cardiac fibrosis, leading to an abnormal accumulation of the ECM components, such as collagen, around damaged heart tissues [45, 46].

Myofibroblast differentiation is a complex and highly regulated process, where biochemical and mechanical factors are interdependent [47]. From a biochemical aspect, the differentiation of cardiac fibroblasts into myofibroblasts is well studied, while the role of mechanical factors remains elusive [48]. When exposed to abnormal mechanical conditions such as strain and ECM stiffness, cardiac fibroblasts can undergo myofibroblast differentiation [49, 50]. A fact worth mentioning within the scope of our investigation is that during the cellular response to heart injury myofibroblasts actively secrete ECM proteins, such as collagen I and III, to replace the damaged myocardium [51]. We previously performed an immunohistochemical and electron microscopy study in order to evaluate the effects of LFN on cardiac collagen and cardiomyocyte ultrastructure [23]. A significant increase of collagens I and III in the ECM was observed. The ultrastructural observation denoted high concentration of collagen in the ECM next to fibroblasts, confirming the pronounced effect of LFN on the connective tissue.

Comparable to the traditional cardiovascular risk factors, experimental and epidemiological evidence substantiates the concept that noise, through auditory and non-auditory effects, may induce activation of different pathways (oxidative stress, vascular dysfunction, autonomic imbalance) that ultimately lead to cardiac fibrosis, adverse ventricular remodeling and arrhythmogenesis [7-12]. It is important to note that non-auditory noise effects (annoyance, sleep disturbance and psychological stress) do not follow the toxicological principle of dosage [7]. Consequently, it needs to be taken into account not simply the accumulated sound energy that causes the adverse effect but also the cognitive perception of the sound, the subsequent

cortical activation and the emotional response. More epidemiological research on LFN and health effects is needed, since the available research is scarce and suffers from methodological shortcomings. A systematic review of observational studies suggest an association between everyday life LFN and IFS components (up to 250Hz) and health effects in the general population, such as annoyance, sleep-related problems, concentration difficulties and headache [52]. However, they underline the inconsistency across studies and the small number of existing observational investigations, precluding a direct comparison with experimental evidence.

This study has some limitations. The number of animals per group was limited, therefore the results should be interpreted cautiously. The significant correlation between the two dependent variables considered in this study, ratio L/W and ratio W/P, as expressed by a Spearman correlation coefficient of 0.705 ($p = 0.005$), would recommend a multivariate approach to the data, in order to account for the effect of the association between variables on type I error. However, given the reduced dimensions of the groups, it is not recommended to assess the multivariate normality and homogeneity of variance-covariance assumptions, in view of the reduced power of the corresponding tests. In these conditions, the Mann-Whitney test has been used to compare the two groups for ratio L/W and ratio W/P variables, with the Bonferroni correction $\alpha^* = 0.05/2 = 0.025$. For the reasons mentioned above regarding the correlation between the dependent variables and group dimension, a non-parametric approach to the data was implemented in the comparison between 3 groups. The Kruskal-Wallis test has been applied with the same Bonferroni correction to the significance level, $\alpha^* = 0.025$, and *post-hoc* comparisons between the groups were conducted for ratio W/P using the Mann-Whitney test, at the $0.025/3 = 0.0083$ significance level, to control for inflation of type 1 error. Also, experimental noise stress models are scarce and, at the present time, a well-defined morphological cardiac model to study the consequences of IFS exposure does not exist. There is a lack of consensus regarding the cardiac cell composition, including fibroblasts, in mammals, with potential variations between species that also depends on the age [53]. Concerning the characteristics of noise, public health research uses A-weighting method to measure noise and focus on sound pressure level, disregarding frequencies. We believe that both sound frequency and intensity are key factors. So far, we investigated the structural modifications in the rat myocardium induced by high sound pressure noise of different wavelengths, from industrial to IFS. Addressing these important questions at the mechanistic level in animals may help provide directions for studies in humans, as more epidemiological research is imperative.

5. Conclusions

Infrasound exposure induces coronary perivascular fibrosis that differs under corticosteroid administration, which raises the possibility of an underlying inflammatory mechanism. The importance of noise in perturbation of inflammatory factors needs to be further investigated.

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5. ATRIAL FIBROSIS AND DECREASED CONNEXIN 43 IN RAT HEARTS AFTER EXPOSURE TO HIGH-INTENSITY INFRASOUND

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Atrial fibrosis and decreased connexin 43 in rat hearts after exposure to high-intensity infrasound

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Abstract

Background: Noise is an important environmental risk factor. Industrial environments are rich in high-intensity infrasound (hi-IFS), which we have found to induce myocardial and coronary perivascular fibrosis in rats. The effects of exposure to IFS on the ventricles have been studied, but not on the atria. We hypothesized that rats exposed to hi-IFS develop atrial remodeling involving fibrosis and connexin 43, which we sought to evaluate. **Material and Methods:** Seventy-two Wistar rats, half exposed to hi-IFS (120dB, <20Hz) during a maximum period of 12 weeks and half age-matched controls, were studied. Atrial fibrosis was analyzed by Chromotrope-aniline blue staining. The immunohistochemical evaluation of Cx43 was performed using the polyclonal antibody connexin-43m diluted 1:1000 at 4ºC overnight. Digitized images were obtained with an optical microscope using 400× magnifications. The measurements were performed using *image J software*. A two-way ANOVA model was used to compare the groups. **Results:** The mean values of the ratio "atrial fibrosis / cardiomyocytes" increased to a maximum of 0.1095±0,04 and 0.5408±0,01, and of the ratio "CX43 / cardiomyocytes" decreased to 0.0834±0,03 and 0.0966±0,03, respectively in IFS-exposed rats and controls. IFS-exposed rats exhibited a significantly higher ratio of fibrosis ($p<0.001$) and lower ratio of Cx43 ($p=0.009$). **Conclusion:** High-intensity infrasound exposure leads to an increase in atrial interstitial fibrosis and a decrease in connexin 43 in rat hearts. This finding reinforces the need for further experimental and clinical studies concerning the effects of exposure to infrasound.

Keywords: Infrasound, atrial fibrosis, connexin 43.

1. Introduction

Noise is an important environmental and occupational risk factor and it is consensual that human exposure to this aggressor can induce systemic damage, thus having an impact on public health. The characteristics of the noise stimulus that may be responsible for inducing this aggression are not fully known. The World Health Organization (WHO) Regional Office for Europe has been particularly concerned with sound pressure level (dB) limits but also acknowledges that low frequency noise (LFN), below 200Hz, represents an environmental problem [1, 2].

Research on the impact of LFN, below 200Hz, and Infrasound (IFS), below 20Hz, established that they are hazardous for the human body, particularly for pressure levels above 120dB [3, 4]. From a physics standpoint, noise and sound are the same, defined as a pressure disturbance that propagates through a material at a speed which is dependent on the material [5]. When considering sound propagation, an obstacle in the sound path will cause its reflection, absorption or transmission through the object. In general, these three processes depend on the wavelength of the sound. Low frequency noises, by having longer wavelengths, are likely more transmitted through the body wall, thus affecting internal systems and organs [3-6]. Furthermore, high intensity-LFN and IFS can induce resonance responses in body cavities [4]. The overall range of human body resonant frequencies was found to be from 2 to 16Hz [7], which is almost the exact range of IFS. The displacement between organ and skeletal structures places biodynamic strain on the involved body tissue and it is known to reach its maximum under exposure to noise close to the body's resonant frequency.

Noise affects both auditory and non-auditory systems of humans exposed to it [8]. The type of exposure (continuous, occasional and occupational) and its duration may trigger different responses [9, 10]. According to experimental and epidemiological studies, the non-auditory effects of noise include annoyance, sleep disturbance and psychological stress and affect the cardiovascular system [3, 4, 8]. Other animals also possess inherent specific sound frequencies in certain tissues and organs [11]. Studies on the morphological and biological effects of exposure to different types of noise, from industrial to LFN, in animal models, showed an increased volume of connective tissue and collagen fibres in different tissues and organs [12-14]. In the cardiovascular system of rodents, we reported the development of perivascular fibrosis around the coronary arteries of rats exposed to industrial noise [15, 16] and documented a significant fibrotic development in ventricular myocardium among rats submitted to LFN [17, 18], together with a possible ventricular gap junction remodeling [19]. We also found that IFS exposure induces coronary perivascular fibrosis [20].

The purpose of our study was to test the hypothesis that different periods of high intensity infrasound exposure (120dB, <20Hz) can cause development of atrial interstitial fibrosis and modifications of Cx43 in rats, by performing a histological and immunohistochemical evaluation.

2. Material and Methods

2.1 Animals

Following the 3Rs principles [21], this study shares data and resources with a larger study of the effects of infrasound exposure on the pancreatic morphology and function, which was approved by the Animal Welfare Body (ORBEA) of Abel Salazar Biomedical Sciences Institute, University of Porto (Portugal), under the protocol nº 204/2017. Thus, seventy-two male Wistar rats purchased from a Spanish breeder (Charles River Laboratories España, S. A., Spain), aged 16 weeks, weighing 375.95 ± 18.29 g, were selected from the original sample. The postmortem collection of hearts did not alter the approved primary protocol procedures in any way. All the handling and care of the experimental animals was performed by authorized researchers (accredited by the Federation of European Laboratory Animal Science Associations, Category C) and was done in accordance with the EU Commission on Animal Protection for Experimental and Scientific Purposes (2010/63/EU) and with the Portuguese legislation for the same purpose (Decree-Law No. 113/2013). The rats were housed in 42 x 27 x 16cm polypropylene cages with a steel lid and had unrestricted access to food (standard commercial chow) and water. A maximum of two rats were kept in a single cage. The same standard house conditions were used throughout the experiment for all the animals.

In the beginning of the study, the seventy-two rats were randomly distributed into two groups. Thirty-six of the rats (group IFS) were continuously exposed to high intensity and very LFN (2-20 Hz/ Lp=120 dB), during a period of 1, 6 or 12 weeks. The remaining thirty-six rats (group CTL) were used as age-matched controls and were kept in a silent environment. Each group was divided into three subgroups with twelve specimens and sacrificed after 1, 6 and 12 weeks. All rats were euthanized following overnight fast by inhalation of gaseous carbon dioxide.

2.2 Electroacoustic experiment

The electroacoustic experiment was described before [20]. In summary, a pseudo-random waveform in the 2 to 20Hz decade band was designed with Matlab based on a bandpass-filtered 30-s maximum length sequence segment and the resulting acoustic pressure waveform involved an average sound pressure level of 120dB with a tolerance of ±3dB.

2.3 Histology

After exsanguination of the sacrificed rats by puncture of the caudal vena cava, hearts were excised, sectioned transversely from the ventricular apex to the atria and routinely processed for light microscopy. The atrial fragment from each heart was selected for the study. Paraffin-embedded sections (3.5µm thickness) of the atrial tissue samples were made and dyed according to Chromotrope Aniline Blue (CAB) staining protocol. For the histological analysis of fibrosis, digitized images were obtained with an optical microscope (Leica® MZ6), equipped with a digital camera (Leica® DF 290HD), under 400× magnification (figure 1).

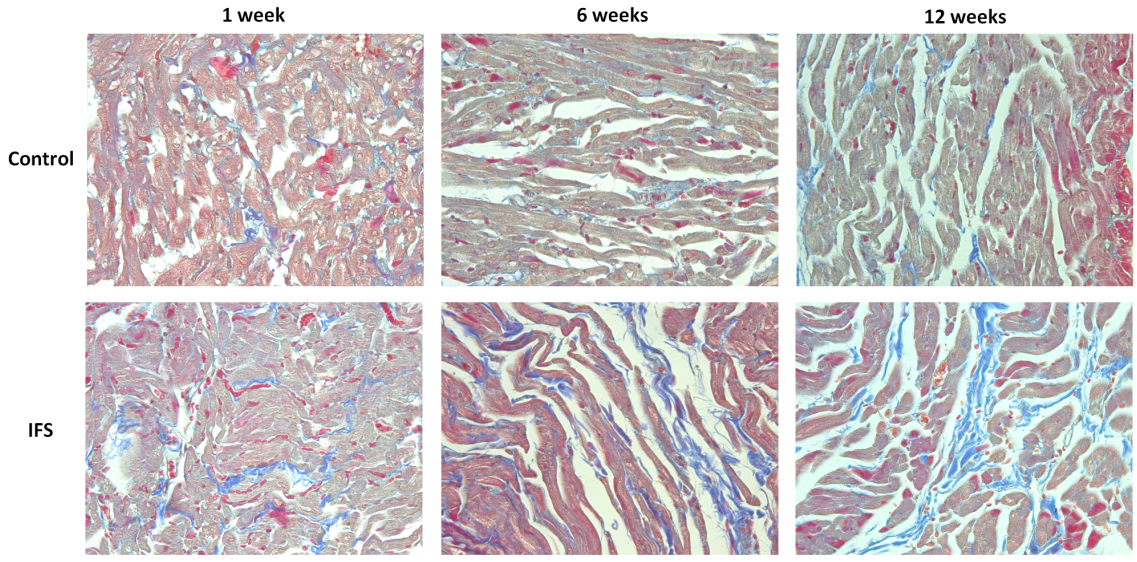

Figure 1. Atrial interstitial fibrosis after high-intensity IFS (120dB, <20Hz) exposure. Examples of CAB-stained atrial sections from control and IFS-exposed rats at 1, 6 and 12 weeks [CAB, 400x]. Note de prominent interstitial fibrosis in IFS-exposed animals. IFS – Infrasound. CAB - Chromotrope Aniline Blue.

For each atrium, three random images of equal area containing fibrosis in the absence of any arterial vessel were selected and analyzed using the *image J software* (National Institutes of Health, Bethesda, MA, USA). For each image, the blue pixel content was measured relative to the total tissue area (the non-staining sections in interstitial spaces were excluded from quantification), using a color deconvolution method [22], and the ratio of fibrosis area to atrial cardiomyocytes area was calculated. The mean ratio of the three images was obtained and used for the comparison between the rats. The researchers, including data collectors and data analysts, were blinded to which group the animals belonged to.

2.4. Immunohistochemistry

For the immunohistochemistry study, sections adjacent to those employed for histology were used. Specifically, 3.5μm fixed formalin paraffin embedded tissue sections on charged slides were placed in an oven at 60°C for 30 min. Sections were deparaffinized, then endogenous peroxidase was blocked with 3% hydrogen peroxide distilled water for 10 min at room temperature (RT). After antigen retrieval, the slides were incubated with rabbit polyclonal anti-Cx43 / GJA1 antibody (abcam, ab11370), 1:1000, at 4ºC overnight. Anti-rabbit Real Envision® HRP Polymer was applied and incubated for 30 min at RT. Diaminobenzidine chromogen reagent was applied and incubated. The sections were counterstained with Harris Hematoxylin and finally mounted with a coverslip using Entellan Mounting Medium (Merck, Darmstadt, Germany).

For the quantification of Cx43, a similar method applied for fibrosis was used. For each atrium, three random images of equal area containing Cx43 immunostaining were selected (figure 2) and analyzed using the *image J software*. For each image, a threshold method was used to determine the number of brown pixels corresponding to Cx43 staining relative to the total tissue area (interstitial spaces were excluded), and the ratio of Cx43 area to atrial cardiomyocytes area was calculated and averaged for each animal. As before, the researchers were unaware of which group the animals belonged to.

2.5 Statistical analysis

Data are presented as mean ± SD. A two-way ANOVA model was used to fit the data of the two dependent variables, ratio of atrial fibrosis area / cardiomyocytes area and ratio of Cx43 area / cardiomyocytes area, in the comparison of IFS-exposed animals and group control. A p value <0.05 was considered statistically significant.

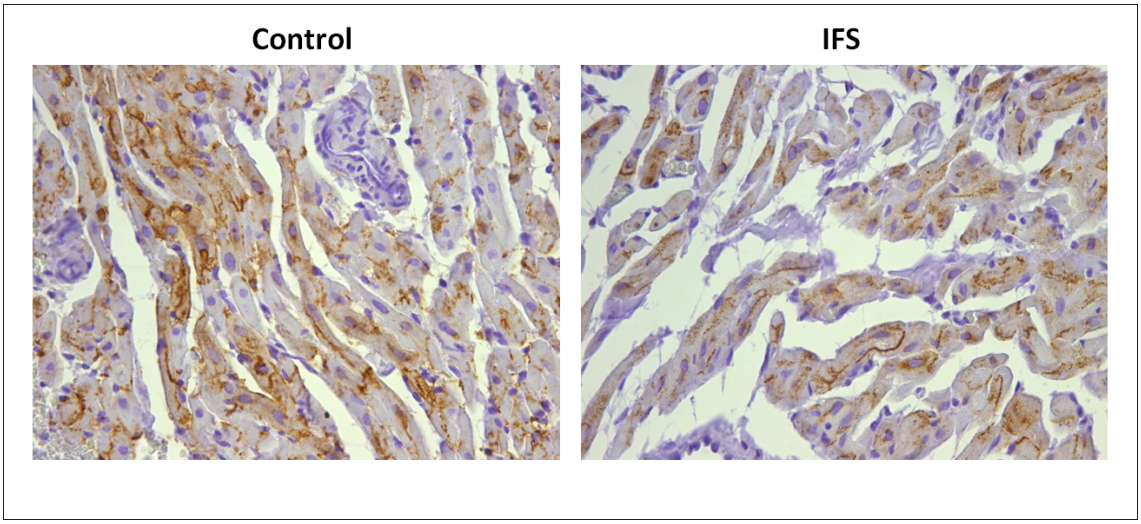

Figure 2. Atrial connexin 43 after high-intensity IFS (120dB, <20Hz) exposure. Examples of atrial sections from control and IFS-exposed rats at 12 weeks [400x]. IFS-exposed animals exhibit lower concentrations of Cx43. IFS – Infrasound.

3. Results

The main results are presented in table 1.

Table 1. Mean ± SD of the two measured outcomes in the two groups

	Time of exposure (weeks)	Group IFS (n = 36)	Group CTL (n = 36)
Ratio of atrial fibrosis area / cardiomyocytes area Mean ± SD	**1**	0.0896 ± 0.04	0.0460 ± 0.03
	6	0.0936 ± 0.03	0.0491 ± 0.01
	12	0.1095 ± 0.04	0.0541 ± 0.01
Ratio of atrial CX43 area / cardiomyocytes area Mean ± SD	**1**	0.1100 ± 0.03	0.1371 ± 0.03
	6	0.0829 ± 0.04	0.1036 ± 0.03
	12	0.0834 ± 0.03	0.0966 ± 0.03

IFS – Infrasound; CTL – Control; SD – standard deviation; Cx43 – Connexin 43

Ratio of atrial fibrosis area / cardiomyocytes area

No interaction between the two independent factors (exposure to IFS and duration of exposure) was observed (p=0.762) and no significant effects on atrial interstitial fibrosis due to time were detected (p=0.272).

Infrasound-exposed animals showed significantly higher ratio of atrial fibrosis area compared to controls (p<0.001), independently of time and with an observed power in excess of 95% (figure 3).

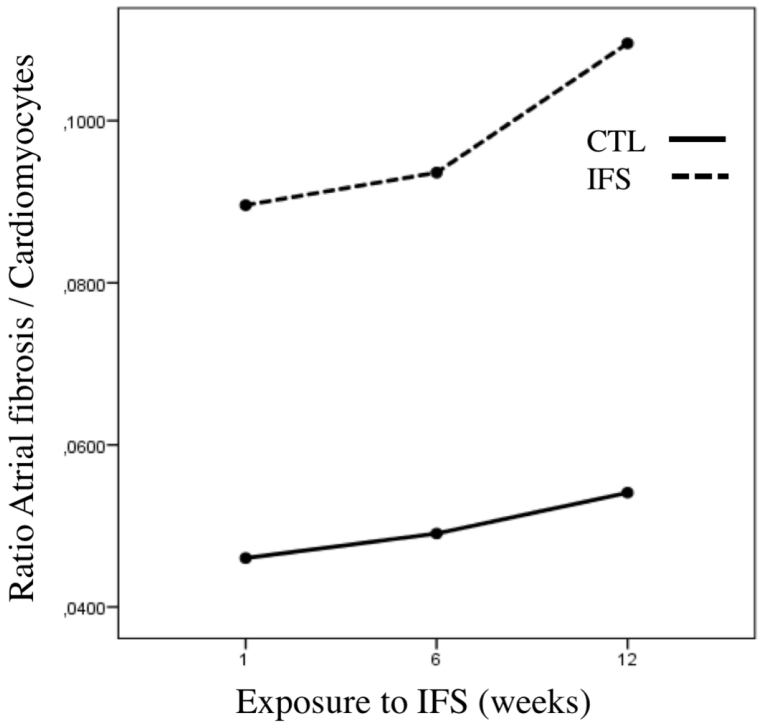

Figure 3. Ratio of fibrosis area to atrial cardiomyocytes area in IFS-exposed and control animals. The ratio was significantly higher in IFS-exposed animals (p<0.001).

Ratio of atrial CX43 area / cardiomyocytes area

There is no interaction between the two independent factors (exposure / time) on their potential effects on the Cx43 modifications (p=0.751). The potential effects of exposure are independent of the potential effects of time.

Exposure to IFS has a significant effect on Cx43 modifications (p=0.009), with IFS-exposed rats showing significantly lower values, independently of time and with an observed power of 75.9% (figure 4).

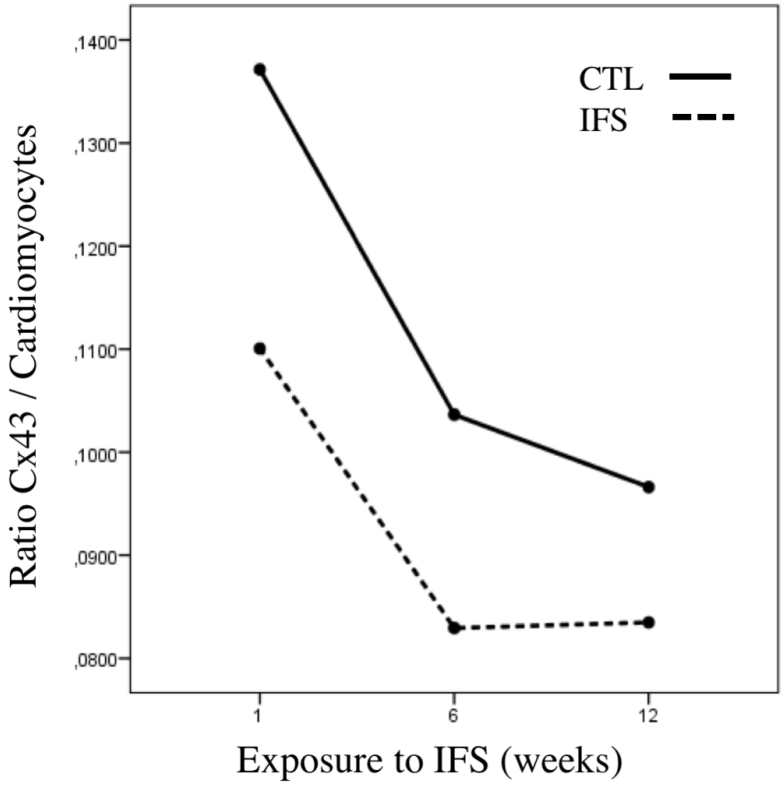

Figure 4. Ratio of Cx43 area to atrial cardiomyocytes area in IFS-exposed animals and group control. The ratio was significantly lower in IFS-exposed animals (p=0.009). Furthermore, Cx43 concentration seems to decrease significantly with time (p=0.001), independently of exposure and with an observed power of 95%.

Moreover, Cx43 concentration decrease significantly with time (p=0.001), independently of exposure to IFS and with an observed power of 95%.

The weight of the rats at euthanasia was considered as a potential covariate in the model for Cx43, since the two variables present a modest but significant bivariate correlation (r = -0.246; p=0.045). However, when included in the model with exposure to IFS and duration of exposure as independent factors, the weight did not present any association with Cx43 nor did the conclusions stated above for this marker change. Therefore, even after controlling for body weight, the effects of exposure and duration of exposure remain virtually unchanged.

4. Discussion

The present study evaluated two specific features of myocardial remodeling in rat heart, interstitial fibrosis and Cx43 modifications, as a consequence of exposure to IFS (120dB, <20Hz) and validated our initial hypothesis that exposure to different periods of high-intensity IFS can lead to structural atrial remodeling. These results are consistent with those of past investigations from our group, also in Wistar rats [12-20], confirming the abnormal proliferation of connective tissue as the main morphological change induced by LFN exposure. In our study, Cx43 also appears to undergo age-dependent loss, as lower concentrations were found among ≥ 22 weeks-old controls compared to younger rats (16 weeks-old), a result that comes in agreement with previously reported data from Watanabe *et al* [23].

High-intensity IFS exposure studies in laboratory animals are scarce but consistently present the deleterious effects of this stressor on the cardiovascular system [11, 24-26]. These experiments were conducted with high-pressure IFS (130dB, 5Hz), in similar conditions to one of our previous studies [20] and to the present one.

Artificial sources of LFN and IFS include industrial installations and low-speed machinery, like diesel engines and wind turbines [4]. As previously mentioned, the WHO Regional Office for Europe recognizes that sound with frequencies below 200Hz represents an environmental problem [1, 2]. It is widely accepted that noise has impact on public health, although the relative contribute of its intensity, frequency content, mean and peak decibel level, as well as the pattern or duration of the exposure, is not well understood. Low-frequency noise is implicated in several adverse biological effects in experimental and epidemiological studies [3, 4], which is partially attributed to the characteristics of strong penetration and less attenuation in long distance propagation.

We do not know by which mechanisms noise induces cardiac fibrotic proliferation in rats. The differentiation of cardiac fibroblasts into more active myofibroblasts, a complex and highly regulated process where biochemical and mechanical factors are interdependent, is the hallmark of cardiac fibrosis [27, 28]. Although the role of mechanical factors remains elusive, cardiac fibroblasts exposed to abnormal mechanical conditions such as strain and extracellular matrix stiffness can undergo myofibroblast differentiation, leading to an abnormal accumulation of the extracellular matrix components, such as collagen, in the heart [29-30].

High pressure levels of LFN can elicit body vibrations and act as mechanical stressor [3, 4, 7, 31]. Humans, as well as other animals, possess inherent specific sound frequencies in certain

tissues and organs in the same range of IFS, below 20Hz [4]. Exposure to vibration close to that resonant frequency range can lead to maximum displacement between the organ and the skeletal structure, placing biodynamic strain on the body tissue involved [7]. We believe this could be among the underlying mechanisms leading to the structural changes we found in our investigations. Comparable to the traditional cardiovascular risk factors, experimental and epidemiological evidence indicates that noise, through auditory and non-auditory effects, may induce activation of different pathways (oxidative stress, inflammation, vascular dysfunction, autonomic imbalance) that ultimately lead to cardiac fibrosis, adverse ventricular and atrial remodeling and arrhythmogenesis [8, 32-35].

The first study reporting an association between residential exposure to road traffic noise and higher risk for developing AF was published in 2016, but this association lost statistical significance after adjustment for air pollution [36]. Two years later, a group of investigators reported the existence of a significant association between environmental noise exposure, including aircraft and road traffic noise, and AF in a large cohort study, involving more than fourteen thousand participants [37]. Furthermore, a third group [38] found an association between long-term exposure to wind turbine noise, known to generate lower frequencies of sound than road traffic [2, 39], and the risk of incidental AF in a large, nationwide cohort of women above age 44. In these studies, the authors propose the indirect / non-auditory pathway of Babisch's noise and stress-reaction model [40] as the most plausible explanation for their findings but AF is an extraordinarily complex arrhythmia involving several pathophysiological mechanisms [41] and there are no studies specifically addressing the pathophysiology of noise-induced AF.

Despite the different underlying mechanisms proposed, the most consistently reported structural change in animals and patients with AF is atrial fibrosis [42, 43]. Among the principal causes of cardiac fibrosis are genetic predisposition, old age, mechanical overload of the heart and myocardial infarction [44]. Cardiac fibrotic remodeling distorts the homogeneous electric substrate and leads to abnormal impulse generation and propagation, representing the most thoroughly investigated mechanism in arrhythmogenesis. But the structural correlate of atrial fibrillation also comprises gap junction remodeling [45-47]. The distribution patterns of connexins are comparable between rat, guinea pig, porcine, bovine and human hearts [48]. In rats, atrial remodeling involving increased fibrosis and altered atrial Cx43 expression consistently lead to higher inducibility of AF [42, 49, 50], regardless of the pathological condition (diabetes, elevated afterload or obstructive sleep apnea), with overlapping findings in humans [51]. From the histopathologic standpoint, it is reasonable to assume that IFS-induced atrial remodeling, with increased fibrosis and decreased Cx43, shares the same

functional relevance. Animal models contribute to our knowledge of arrhythmogenesis. The pathophysiological basis of arrhythmias is not completely understood but different types of arrhythmias share similarities in their basic mechanisms [52].

The main limitation of this study is that we did not address the functional relevance of the atrial remodeling involving fibrosis and Cx43 modifications, leaving open the question of whether it can act as an arrhythmogenic substrate. Fundamental mechanisms can potentially be identified in rats and translated into clinical practice, even considering marked electrophysiological differences in comparison to humans. We consider that this is an initial investigation, as translation of our findings and preclinical studies should be conducted in larger animals and would implicate a different research protocol [52]. There are other minor limitations. First, due to the small size and the curvature of the atria of the rats, it was not possible to obtain sections in which all the muscle fibers were oriented in the same plane for histologic and immunohystochemical analysis. Second, experimental noise stress models are scarce and, at the present time, a well-defined morphological cardiac model to study the consequences of IFS exposure does not exist. There is a lack of consensus regarding the cardiac cell composition, including fibroblasts, in mammals, with potential variations between species that also depends on the age [53]. Finally, concerning the characteristics of noise, environmental studies mostly use A-weighting method to measure noise and focus on sound pressure level, disregarding frequencies. Low-frequency sounds have higher energy than the sounds at mid and higher frequencies and cannot be correctly evaluated using the conventional A-filters [6]. Nonetheless, we believe that both sound frequency and intensity are key factors and should be considered in future research.

5. Conclusions

High-intensity infrasound exposure triggers atrial structural remodeling with increased interstitial fibrosis and decreased Cx43 in rats. The functional consequences of this finding are not known, reinforcing the need for further research concerning the effects of IFS exposure on the heart.

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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6. FINAL DISCUSSION AND CONCLUSIONS

FINAL DISCUSSION AND CONCLUSIONS

This thesis was build up on previous research on the cardiac morphological changes induced by IN and LFN in Wistar rats and addressed important gaps in knowledge that have emerged from studies conducted in the past three decades.

As stated before, LFN and IFS can act as a mechanical stressor, under which exposure the main morphological change induced is a systemic abnormal proliferation of connective tissue affecting several organs and tissues, such as gastric mucosa, lung parenchyma, tracheal epithelia, adrenal cortex, parotid gland, lymphatic and arterial vessels and the heart [1-11].

The relative contribute of noise intensity, frequency content, mean and dB peak level, pattern or duration of the exposure that may be responsible for inducing this aggression is not clarified.

In this project, experiments were conducted sequentially in order to analyze whether structural changes induced by IN exposure extend to the small coronary arteries and arterioles [12], whether IFS leads to the same morphological changes induced by LFN in rat coronaries and if these are influenced by treatment with an anti-inflammatory agent [13] and finally, if the atria of rats exposed to high-intensity IFS are vulnerable to its effects [14].

In the **first study** [12], rats were exposed to IN rich in LFN (<500Hz, ≥90dB), in experimental conditions that tried to simulate the schedule of industrial plant workers, characterized by 8 hours a day, 5 days a week, with weekends in silence. The duration of exposure ranged from 1 to 7 months.

In rats, long-term exposure to LFN (>100dB) causes a focal thickening of the intima and disruption of the internal elastic lamina of medium and large-caliber blood vessels such as the aorta and the femoral artery, but these morphological changes were not observed in the small vessels [7]. In the heart, the coronary arteries of rats exposed to IN showed prominent perivascular tissue and fibrotic development [9] but we did not know whether these structural changes were extensible to the small coronary arteries.

Our results show an increase in the perivascular tissue around the small coronaries and arterioles in rats exposed to IN, in agreement with the findings on the coronary arterial vessels [9]. The maximal effect was observed at three months of IN exposure, with a gradual reduction observed until seven months. The influence of IN over the heart appears to affect equally the right ventricle, septum and left ventricle, as the analysis of data *per* anatomic region did not

demonstrate any particular differences. At seven months of exposure to IN, a statistically significant increase in wall thickness of small coronary arteries was observed in exposed rats, as compared to controls. Such differences were not observed in the coronary arterial vessels [9]. We speculate that IN effects on wall thickening require longer times of exposure and that the arterioles are the first to be affected.

We did not address the functional consequences of these structural changes. The changes in the structure of coronary resistance vessels have been extensively studied under several experimental conditions (arterial hypertension, volume overload-induced cardiac hypertrophy, diabetic cardiomyopathy, chronic renal failure) and were already extended to humans [15-21]. They contribute to myocardial ischemia, left ventricular wall stiffness, systolic and diastolic dysfunction and arrhythmogenicity.

Epidemiological studies have shown that road traffic, aircraft and rail traffic noise have a dose-dependent association with elevated cardiovascular morbidity and mortality [22]. The latest WHO Regional Office for Europe environmental noise guidelines [23] considers cardiovascular disease to be a critical health outcome measure and state the existence of a relative risk of 1.08 [1.01; 1.15] for the occurrence of coronary artery disease, starting at 50 dBA. Moreover, the detrimental impact of noise on patients with chronic coronary syndromes was highlighted for the first time in the 2019 European Society of Cardiology guidelines [24]. Altogether, research into the impact of noise exposure has increased considerably in both quantity and quality in the last years and has gained overdue attention.

The pathophysiological mechanisms by which noise increases the risk of cardiovascular disease have not been fully elucidated and are a topic of ongoing research. The assumption is that noise exposure leads to stress reactions or noise annoyance reactions, which in turn, via activation of the sympathetic nervous system and increased release of stress hormones, favors the development of oxidative stress and inflammatory processes, resulting in disruption of vascular and endothelial function [22].

The **second study** [13] addressed two important questions, as evaluated whether exposure to noise with frequencies below 20Hz can induce coronary morphological changes in rat heart and assessed for the first time the possible influence of an anti-inflammatory agent on these changes. Rats were continuously exposed to high intensity IFS (120dB, <20Hz) for a period of 28 days. The results showed an increase in the perivascular tissue around the coronary arteries in rats exposed to IFS compared to controls. But such differences did not reach

statistical significance in animals treated with dexamethasone, pointing to the existence of a possible underlying inflammatory mechanism.

Until recently, it was presumed that noise below 200Hz required greater sound pressure levels in order to elicit toxicological effects on humans and animals [25, 26]. Historically, high intensity IFS below 20Hz was thought to be of much less significance than LFN in the 20-200Hz range at the same pressure level [25]. For sound pressure levels above 120dB IFS is considered hazardous to the human body [26].

High sound pressure levels can be harmful to the cochlea and cause hearing loss, raising the question of other noise effects being secondary to direct auditory damage. Some investigators explored the effects of low decibel noise, <90dB, thought to cause mainly non-auditory effects to animals such as stress reactions [27, 28]. But the results are conflicting, as one group claims that noise below 90dB can elicit cardiac protective effects [27], while the other group reports detrimental changes in vascular endothelial function, vascular production of reactive oxygen species and increased blood stress hormones and biomarkers of inflammation [28]. It seems that sound pressure level alone may not explain all the detrimental health effects of noise. The World Health Organization (WHO) Regional Office for Europe acknowledges that LFN, below 200Hz, represents an environmental problem [23] and has included wind turbines - known to generate lower frequencies of sound than road traffic [29] - as a new noise source.

We believe that both sound pressure level and frequencies are important to explain the structural changes induced by noise that we found in our investigations. Sound is a physical agent and when encounters an object or obstacle in its path suffers reflection, absorption or transmission through the object, three processes that generally depend on the wavelength of the sound. Since low frequency noises have longer wavelengths, they are likely more transmitted through the body wall, affecting internal systems and organs as a consequence [26, 30-32]. Furthermore, high intensity-LFN and IFS can elicit body vibrations and act as mechanical stressor [26, 31, 33, 34]. Humans, as well as other animals, possess inherent specific sound frequencies in certain tissues and organs in the same range of IFS [26]. The displacement between organ and skeletal structures places biodynamic strain on the involved body tissue and it is known to reach its maximum under exposure to noise close to the body's resonant frequency [33].

Data on the hypothetical noise-induced pathway involving inflammation is limited [35]. As previously mentioned, the common finding in the noise experiments conducted by our group was the perivascular and myocardial fibrotic development in the absence of inflammatory cells

[8, 9, 11, 12]. In the present study, we included a group of IFS-exposed animals treated with dexamethasone that did not show significant differences when compared to controls. As a result, we have to consider a potential underlying inflammatory mechanism, despite the need of further investigation.

A systematic review of observational studies suggests an association between everyday life LFN and IFS components (up to 250Hz) and health effects in the general population, such as annoyance, sleep-related problems, concentration difficulties and headache [36]. However, they underline the inconsistency across studies and the small number of existing observational investigations, precluding a direct comparison with experimental evidence. Thus, more research is needed to clarify the public health burden originating from LFN and IFS.

Finally, the **third study** [14] tested the hypothesis that different periods of high intensity IFS exposure (120dB, <20Hz), from 1 to 12 weeks, can lead to atrial structural remodeling involving the development of interstitial fibrosis and modifications of Cx43 in rats. The results are consistent with those of past investigations from our group [1-13] and confirm the abnormal proliferation of connective tissue as the main morphological change induced by LFN and IFS exposure. After extensive study of the ventricles, it was the first time that the atria were studied under such an electroacoustic experimental protocol.

We do not know by which mechanisms noise induces cardiac fibrotic proliferation in rats. Cardiac fibroblasts exposed to abnormal mechanical conditions such as strain and extracellular matrix stiffness can undergo myofibroblast differentiation, leading to an abnormal accumulation of the extracellular matrix components in the heart [37, 38]. As stated before, high pressure levels of LFN can elicit body vibrations and act as mechanical stressor [26, 31, 33, 34], which could be among the underlying mechanisms leading to these structural changes.

In the past years, we witnessed the emergence of increasing evidence for an association between noise exposure and the incidence of AF [39-41]. In these studies, the authors propose the indirect / non-auditory pathway of Babisch's noise and stress-reaction model [42] as the most plausible explanation for their findings but AF is an extraordinarily complex arrhythmia involving several pathophysiological mechanisms [43] and there are no studies specifically addressing the pathophysiology of noise-induced AF.

Despite all proposed mechanisms, fibrosis remains the cornerstone of atrial pathology in patients with AF, with several studies providing evidence for an association between cardiac fibrosis and gap junction remodeling that could contribute to the arrhythmogenic substrate [44,

45]. In rats, atrial remodeling involving increased fibrosis and altered atrial Cx43 expression consistently lead to a higher inducibility of AF [45-47], regardless of the pathological condition (diabetes, elevated afterload or obstructive sleep apnea), with overlapping findings in humans [48].

In our study, we did not address the functional relevance of the atrial remodeling, as translation of these findings should be conducted in larger animals. Nonetheless, from the histopathologic standpoint, it is reasonable to assume that IFS-induced atrial remodeling, with increased fibrosis and decreased Cx43, shares the same functional relevance. That leaves open the question of whether IFS-induced atrial remodeling correlates to arrhythmogenic substrate in atrial fibrillation associated with environmental noise, which should encourage further experimental studies concerning the effects of infrasound on the heart.

Overall, high intensity LFN and IFS induced cardiac morphological changes in rats whether the pattern of exposure was intermittent, as in the first study, or continuous, as in the second and third studies. Periods of exposure as short as one week were enough to elicit the development of myocardial interstitial fibrosis compared to controls. Nonetheless, for certain cardiac structures, such as the small coronaries and arterioles, some structural changes (wall thickening) required longer times of exposure, up to seven months. Consequently, the effects of acute and chronic exposure to noise may be different and future research should address this issue adequately.

Animal models may contribute to our knowledge of pathophysiology. An ideal animal model should accurately resemble human cardiovascular anatomy and physiology, allow easy housing and breeding at reasonable costs and be accessible for standard experimental techniques, but the combination of all of the above characteristics in a single species is very hard to find [49].

In our investigations to identify cardiac structural changes induced by LFN and IFS we selected the Wistar rat as the experimental animal model. They are widely available in large numbers and at fairly low costs, our facilities allow standardized housing, the electroacoustic experiments are fairly easy to perform in this animal and we have a vast experience from previous experimental studies involving this species. On the basis of species-specific advantages, it seemed to be the most convenient animal to study our particular scientific questions. However, rat hearts are small and their physiological properties differ from those of humans. Therefore, investigations that require specialized equipment (for example, assessment of the heart rhythm by electrocardiogram) might be challenging in the rat.

As a result, the main limitation of the studies included in this thesis is that we did not address the functional relevance of the structural changes induced by LFN and IFS. Fundamental mechanisms can potentially be identified in rats and translated into clinical practice, even considering marked physiological differences in comparison to humans [49]. But in the future, we consider the use of larger animals and a different research protocol in order to translate our findings into preclinical studies and clinical practice.

Also, there is a lack of consensus regarding the cardiac cell composition, including fibroblasts, in mammals, with potential variations between species that also depends on the age [50].

Finally, in respect of the characteristics of noise, environmental studies mostly use A-weighting method to measure noise and focus on sound pressure level, disregarding frequencies. Low-frequency sounds have higher energy than the sounds at mid and higher frequencies and cannot be correctly evaluated using the conventional A-filters [32]. We propose that sound frequency assessment should be included in future research.

In conclusion:

1. In the heart, exposure to industrial noise, characterized by high pressure levels and a wide spectrum of wavelengths that includes LFN, can induce an increase in the perivascular tissue of rat small coronary arteries, with significant development of periarterial fibrosis. The effects are similar to those observed on large coronary arteries but chronic exposure can also cause thickening of the small coronary vessel wall, not observed in the first case.

2. High pressure level IFS exposure, just as LFN, induces coronary perivascular fibrosis in rats.

3. The existence of an underlying inflammatory mechanism should be considered, as the administration of an anti-inflammatory agent interferes with the development of IFS-induced perivascular fibrosis.

4. High pressure level IFS exposure effects involve the atria of rats, in addition to the effects previously studied in the ventricles, valves, pericardium and coronary vasculature.

5. The effects of exposure to LFN and IFS seem to be independent of its pattern (intermittent vs continuous) but chronic exposure can elicit additional structural changes, not observed in the acute setting.

6. We propose that both sound frequency and pressure level are key factors to explain the toxicological effects induced by noise and should be considered in future research.

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New observations on cardiac morphological changes induced by low-frequency noise and infrasound in rats

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