Effects of Large Pressure Amplitude Low Frequency Noise in the Parotid Gland Perivasculo-Ductal Connective Tissue

Efeitos do Ruído de Baixa Frequência de Alta Amplitude no Tecido Conjuntivo Perivasculo-Ductal da Glândula Parótida

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ABSTRACT

Introduction: In tissues and organs exposed to large pressure amplitude low frequency noise fibrosis occurs in the absence of inflammatory signs, which is thought to be a protective response. In the parotid gland the perivasculo-ductal connective tissue surrounds arteries, veins and the ductal tree. Perivasculo-ductal connective tissue is believed to function as a mechanical stabilizer of the glandular tissue.

Material and Methods: In order to quantify the proliferation of perivasculo-ductal connective tissue in large pressure amplitude low frequency noise-exposed rats we used sixty Wistar rats which were equally divided into 6 groups. One group kept in silence, and the remaining five exposed to continuous large pressure amplitude low frequency noise: g1-168h (1 week); g2-504h (3 weeks); g3-840h (5 weeks); g4-1512h (9 weeks); and g5-2184h (13 weeks). After exposure, parotid glands were removed and the perivasculo-ductal connective tissue area was measured in all groups. We applied ANOVA statistical analysis, using SPSS 13.0.

Results: The global trend is an increase in the average perivasculo-ductal connective tissue areas, that develops linearly and significantly with large pressure amplitude low frequency noise exposure time ($p < 0.001$).

Discussion: It has been suggested that the biological response to large pressure amplitude low frequency noise exposure is associated with the need to maintain structural integrity. The structural reinforcement would be achieved by increased perivasculo-ductal connective tissue.

Conclusions: Hence, these results show that in response to large pressure amplitude low frequency noise exposure, rat parotid glands increase their perivasculo-ductal connective tissue.

Keywords: Fibrillar Collagens; Connective Tissue; Parotid Gland; Rats.

RESUMO

Introdução: Em tecidos e órgãos expostos a ruído de baixa frequência de alta amplitude ocorre fibrose na ausência de sinais inflamatórios, que se pensa ser uma resposta protetora. No tecido conjuntivo perivasculo-ductal da glândula parótida seguem artérias, veias e a árvore ductal. Crê-se que o tecido conjuntivo perivasculo-ductal funcione como um estabilizador mecânico do tecido glandular.

Material e Métodos: Para quantificar a proliferação de tecido conjuntivo perivasculo-ductal em ratos expostos a ruído de baixa frequência de alta amplitude foram utilizados 60 ratos Wistar igualmente divididos em seis grupos. Um grupo mantido em silêncio, e os restantes 5 expostos a ruído de baixa frequência de alta amplitude continuamente: g1-168h (1 semana); g2-504h (3 semanas); g3-840h (5semanas); g4-1512h (9 semanas) e g5-2184h (13 semanas). Após a exposição, as parótidas foram removidas e o tecido conjuntivo perivasculo-ductal foi medido em todos os grupos. Foi efectuada análise estatística com ANOVA por SPSS 13.0.

Resultados: A tendência é um aumento global das áreas do tecido conjuntivo perivasculo-ductal, que se desenvolve de forma linear e significativa com o tempo de exposição ($p < 0,001$).

Discussão: Tem sido sugerido que a resposta biológica à exposição ao ruído de baixa frequência de alta amplitude está associada à necessidade de manter a integridade estrutural. O reforço estrutural seria conseguido através do aumento do tecido conjuntivo perivasculo-ductal.

Conclusões: Assim, estes resultados mostram que o tecido conjuntivo perivasculo-ductal aumenta em resposta à exposição ao ruído de baixa frequência de alta amplitude.

Palavras-chave: Glândula Parótida; Ratos; Tecido Conjuntivo.

INTRODUCTION

Mechanical stressors have long been known to cause morphologic cellular changes[1-3] and organic alterations with functional repercussion[4-6] Large pressure amplitude low frequency noise (LPALFN) – sound pressure >90dBL bandwidth <500Hz – which is present in professional, residential and leisure environments, is a powerful mechanical stressor[7] that causes degenerative cellular changes and organic alterations,[8,9] both in humans and animals.

The abnormal proliferation of connective tissue has been pointed out as one of the main consequences of the exposure to LPALFN in the biologic tissues.[9,10] The lesions, which correspond to fibrosis, occur with surprisingly absence of inflammatory signs.[8,10]

The respiratory system[12-17]; the cardiovascular system[18-20]; or the digestive system,[21-24] have been shown to develop fibrosis when exposed to LPALFN.

The proliferation of components of the extracellular matrix fibrosis is thought to be not only a degenerative sign

– associated to the immunological[25-27] and genotypical alterations[28,29] – but also a protective response of the tissues and organs, by increasing the production of elements with structural role and viscoelastic properties, to the impact of such a strong mechanical stress.[17]

In previous studies on the parotid gland we showed microscopical degenerative cellular alterations, proliferation of perivasculo-ductal connective tissue (PVDCT) and vascular lesions with qualitative and quantitative repercussions on the salivary gland function.[24]

In the parotid gland, the PVDCT surrounds the arteries, the veins and the ductal tree and is thought to have functions as a mechanical stabilizer of the glandular tissue.[30]

Our aim is to quantify the proliferation of PVDCT induced by different exposure times to LPALFN.

MATERIALS AND METHODS
Animals

The Wistar rats used in this study were acquired from Charles River Laboratories, Barcelona Spain, males and females, ages ranging between 8 and 12 months, average weights 300g (SD ± 91g), caged in groups of 2 or 3 with the same gender, with no limits to their movements, and exposed to cycles of 12 hours light/dark. The animals were randomly divided in 6 groups of 10 animals and treated differently. Rats assigned to groups 1 to 5 were submitted to continuous LPALFN for: g1–168h (1week); g2–504h (3 weeks); g3–840h (5 weeks); g4–1512h (9 weeks); g5–2184h (13 weeks). Group 6 was kept in similar laboratory conditions but in silence for control purposes.

All the animals (control and exposed) were fed with the same standard rat food, had unrestrained access to water, and were treated according to the EU directive on Animal Protection for Experimental and Scientific Purposes (86/609/CE) and also according to the Portuguese laws in that concern.

The animals were sacrificed at the end of the exposure periods by a blow to the head followed by cervical dislocation; at the same time two animals from the control group were also sacrificed. Then an incision was made in the upper part of the animal neck and parotid glands were surgically removed, according to the protocol for dissection of cervical structures.[31]

After surgical removal, one of the parotid glands was fixated in formalin, embebed in paraffin and sectioned for light microscopy. Sections were stained with hematoxylin-eosin (HE).

Large Pressure Amplitude Low Frequency Noise exposure

The cages were placed in an isolated compartment, measuring 217×211×195 cm, in front of a noise generator, consisting of a subwoofer Magnat xtc 1200 (Magnat, Germany) that reproduced, continuously, a sound signal, previously recorded, consisting of white noise, amplified and frequency filtered, by a computer Compaq (Pentium 133 MHz) (Compaq, USA) and a QSC amplifier (QSC Audio Products, Inc., USA), creating an acoustic environment rich in low frequency components The noise produced was measured with a digital spectral analyzer, B&K 2144. The spectral analysis was similar in the entire compartment and levels were above 90 dB in the frequencies ranging from 50 to 500 Hz as shown in previous studies by Oliveira et al.[24]

PVDCT analysis/measurement

We undertook an analysis of variance with an ordinal factor in order to determine whether the time of exposure to LPALFN (hours of exposure) had a significant effect on the dependent variable defined by us - the area of PVDCT. For that purpose we've made blinded measures, as many as possible for each HE section, for an ordinal absolute value, the area of PVDCT in all groups. The measurements were made with image analysis software (Optimas, MediaCybernetics) on images captured with a NIKON D100 coupled to an optical microscope (Leica) with magnification ×100. The areas were chosen regardless of their form and taking into consideration the following criteria: a) the presence of vessels and tubules within their boundaries; b) being surrounded by glandular parenchyma.

Statistical data analysis

For the purpose of multiple comparisons between groups we used ANOVA analysis. Data analysis was performed with SPSS 13.0.

Table 1 – Mean areas of PVDCT with exposure time and SD within groups

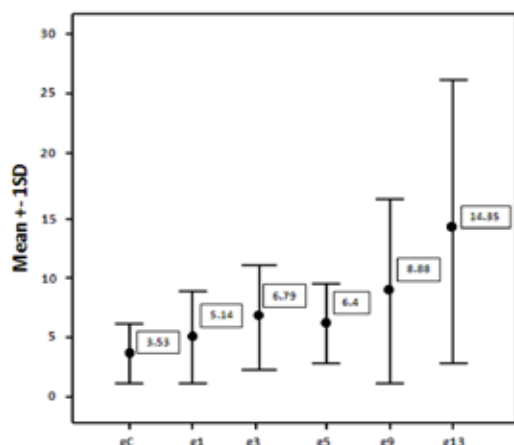| Group	Exposure to LPALFN	Number of measures	Mean of areas	SD
gC	Ø	46	3.5335	2.49288
g1	168h	46	5.1433	3.94804
g2	504h	39	6.7938	4.32579
g3	840h	33	6.4015	3.23013
g4	1512h	41	8.8795	7.46172
g5	2184h	42	14.3486	11.65720

Figure 1 – Evolution of mean PVDCT with time of exposure to LPALFN (*p* < 0.001).

RESULTS
Morphometric analysis

Table 1 and its graphic representation (Fig. 1) show that the areas of PVDCT have a growing change to the g2, 3 weeks, decreasing slightly in the g3 (5 weeks), to grow again until the g5 (13 weeks) (Fig. 1).

As is well known, the use of One-Way ANOVA for group comparisons requires that the data have certain properties, namely normal distribution within groups and homogeneity of variances between groups; therefore, the validity of these assumptions was checked prior to the data analysis, using the Shappiro-Wilk test of normality and the Levene test for variances. These procedures showed that the two assumptions were violated, in which case the Welch ANOVA is preferable for the purpose of group comparisons. The results revealed the existence of significant differences in the mean PVDCT between groups, with *p* < 0.001 (Table 2).

In these conditions, multiple comparisons between groups were conducted using the Tamhane test, which is an appropriate post-hoc test in the case of unequal variances. It was found that the control group shows significant differences when compared to the other groups. We've also found that there are significant differences in the groups exposed to the LPALFN, with the exception of the g4 (9 weeks of exposure to LPALFN) which only shows a significant difference towards the control group. The g5 (13 weeks of exposure to LPALFN) is the one that presents the largest differences relative to the other groups exposed to the LPALFN and seems to indicate a trend for higher growth areas of PVDCT with growing exposure to the LPALFN. Comparing groups g2 (3 weeks) and g3 (5 weeks of exposure to the LPALFN) we can see that there is a stabilization of the average areas of PVDCT (*p* = 1.000). Finally, a contrast analysis was performed to see if there was an increasing linear trend in areas across the groups (Table 3).

The results presented on the last table show that the global trend for areas of PVDCT is increasing, and that the average area of PVDCT increases linearly and significantly with time of exposure to LPALFN (*p* < 0.001).

DISCUSSION

Alterations attributed to LPALFN are secondary to the degenerative cellular changes; to fibrosis[8,10]; immunological[25,26] and genotypical alterations[28,29] and vascular lesions that occurs in small, medium and large caliber vessels.[19,20] The fibrotic processes are characterized by an abnormal and abundant deposition of extracellular matrix components.[32] They have a multifactorial nature and result of a long-term activation of fibroblasts in the affected organs[33] in response to chronic or inflammatory stimuli,[34] occurring also in senescence, in response to ischemia or in neuroendocrine changes.[35] In the case of fibrotic proliferation attributed to LPALFN, the described alterations occur in the absence of inflammatory signs.[10] In our study we did not

Table 2 – Welch test

	Statistic	df1	df2	Sig.
Welch	12.308	6	116.544	< 0.001

Asymptotically F distributed.

Robust Tests of Equality of Means - Area

Table 3 – ANOVA (contrast analysis) Area

			Sum of Squares	df	Mean Square	F	Sig.
Between Groups	(Combined)		4542.024	6	757.004	10.238	0.000
	Linear Term	Weighted Deviation	4320.384	1	4320.384	58.428	0.000
			221.640	5	44.328	0.599	0.700
Within Groups			20408.408	276	73.944		
Total			24950.432	282			

ANOVA - Area

identify cells or signs of any inflammatory process.

The absence of inflammatory signs makes the situation unusual from the point of view of conventional medical concepts, since processes of fibrosis are usually accompanied by inflammation.[10] However there are descriptions of clinical fibrotic processes in the absence of inflammatory signs. The idiopathic pulmonary fibrosis is an example where there is fibroblastic proliferation and remodeling of the extracellular matrix without inflammation that leads to an irreversible distortion of the architecture of the lung.[36]

In the parotid gland there are two populations of collagen fibrils: those associated with fibroblasts and those present in the epithelium of excretory ducts and blood vessels. There is no significant difference in the thickness of the two types of collagen fibrils, which are separated only by a small cluster of elastic fibers.[30] According to Hosoyamada et al. the location of the collagen fibrils in laminae densae of the basement membrane indicates that the epithelial cells synthesize and secrete collagen fibrils,[30] as is the case of the fibrous layer of the liver[37] - leaflet of Glisson -, or the glomerular capsule of Bowman.[38] The production of fiber-forming collagen fibrils type I, III and V, was also detected in several types of epithelia in experimental models of fibrosis.[30]

In intestinal fibrosis associated with radiation, the expression of connective tissue growth factor (CTGF) is increased,[39] and in vitro isolation of intestinal muscle cells also exposed to radiation[40] showed a high concentration of constituent CTGF and a collagen increased production capacity. Bourgier et al. were able to verify that the path Rho / Rho kinase is related to the activation of CTGF, and that its blockade reduced the fibrogenic differentiation.[40]

We have to call up the attention to the references found in relation to the regulation of gene over-expression of molecules such as collagen, in particular by growth factors, in diseases related to mechanical stress, resulting from increased external forces applied to tissues, and mechanical changes in the actin cytoskeleton of the cells. These forces are significantly increased in several conditions such as hypertension, the organic obstruction or hemodynamic overload.[6] In these conditions the cellular components of organs, particularly fibroblasts, endothelial cells and smooth muscle cells are subjected to a mechanical stress that goes beyond what happens under normal conditions.[41] The transmission of such forces to cells in organs such as blood vessels causes a production of growth factors, cytokines or hormones that leads to hypertrophic, hyperproliferative or fibrotic responses.[41] Somehow the impact caused by mechanical vibration or by direct LPALFN may have common paths on a physical point of view with what happens in some of the diseases in which abnormal mechanical stimuli are applied cyclically to the tissues. Consistent with these considerations is that in vitro studies demonstrated that mechanical forces applied to cells, for example fibroblasts, result in profound histomorphometric, phenotypic and functional changes.[2,4,5] In parotid gland exposed to LPALFN we have seen morphometric changes. Oliveira et al proved the existence of functional changes, with reduction in saliva se-

cretion and its qualitative change.[24]

The apparent stabilization of fibrosis caused by LPALFN on g2 to g3 may indicate an adaptive mechanism in which compensatory signaling pathways are activated to enable the gene transcription to go back to normal levels.[42] In the same line is the statement of Reis Ferreira et al that, unlike the fibrotic proliferation in response to an inflammatory stimulus, the tissue exposed to LPALFN, particularly in the airways, seems to reflect a structural reinforcement in order to assimilate the abnormal vibration stress.[17] This structural reinforcement would be achieved by mass production of collagen.[17] In the parotid gland structural reinforcement would be achieved by increased PVDCT. The PVDCT attached to the arteries, veins and the ductal tree is thought to function as mechanical stabilizer of the glandular tissue.[30] Verrecchia et al reported that in fibrosis caused by chronic stimulation, the tissue injury and the attempt of regeneration process are implicated.[32] Thus, in the parotid gland, the period from the 3rd to the 5th weeks of exposure to LPALFN, could be a recovery response in the stroma, which tends to imbalance with the continued application of mechanical stimulation. Also Kisseleva et al. reported that despite the fibrogenesis is a repair mechanism it tends to imbalance with extensive deposition of the matrix proteins and fibrosis with the chronicity of the stimulus on the tissue.[43]

It seems important at this stage to discuss the mechanisms that lead to fibrosis found in the gland and caused by LPALFN, trying to understand the transmission of mechanical energy to the cell, the concept of mechanotransduction; this was associated to the vibroacoustic disease and biological effects of LPALFN as the pathway by which the sound pressure damages the cells and tissues.[10] A starting point for the interpretation of this recent concept in the cell biology that may have an application in the area of biological effects of LPALFN is the interesting experience of Naruse et al. that used low-intensity pulsed ultrasound to accelerate the repair of fractures and distraction osteogenesis.[44] The authors attributed the osteoblastic differentiation to mechanical stimuli, noticing that many physical forces may have consequences in the micro-environment of each cell. The authors also suggested the involvement of integrins in this process at the level of focal adhesions, which are a connection between the matrix and the cells.[3] The integrins seem to be the primary route for the transmission of forces into the cell and are seen as candidates for the starting of mechano-sensitive events.[45] Munger et al demonstrated that bleomycin, a known fibrotic agent,[46] didn't cause pulmonary fibrosis in mice deficient in integrin αvβ6, despite the intense inflammatory reaction.[47]

Mechanotransduction is the mechanism that converts physical stimuli into biochemical signs and integrates these signs into cellular responses at the molecular level.[3] We assume that in intact tissues the cells react to mechanical stimuli with molecular responses that aim to protect their integrity. These forces on the cells may be applied in many different ways provided they have a sufficient magnitude to cause a biological response in the cell.[45] The response of

endothelial cells to mechanical stress caused by fluid pressure is the best studied.[48] It is known the contribution of this kind of mechanical stimulus in the induction of endothelial cell proliferation[49]; it is also known that the critical level of force to initiate various biological responses in cells is approximately 1 Pascal.[50]

Although we cannot make a direct comparison with the processes mentioned in the previous paragraph we should not forget the conclusion of Huang et al: despite the apparent complexity of the process of cell mechanotransduction is likely that cells stimulated in different ways can be activated by similar molecular mechanisms.[45]

CONCLUSIONS

We can conclude that exposure to LPALFN causes PVDCT fibrosis. This fibrosis is significant from the first week of exposure and increases with the increase of exposure time. LFN may activate mechanisms of cellular mechanotransduction that we intend to characterize in future studies.

CONFLICT OF INTERESTS

None stated.

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None stated.

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