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ORIGINAL ARTICLE

Low-frequency noise effects on the parotid gland of the Wistar rat

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BACKGROUND: Long-term low-frequency noise (LFN) (≤500 Hz) exposure is known to cause extracellular matrix proliferation by fibrosis in the absence of inflammatory signs.

AIM: We have examined the morphofunctional alterations of the parotid glands of Wistar rats exposed to LFN. The hypothesis is that the parotid glands are a target for LFN-induced lesions.

MATERIALS AND METHODS: Ten rats were exposed to LFN for 2184 consecutive hours while 10 rats were kept under similar conditions but in silence for control purposes. The parotid glands were surgically removed. One gland was sectioned and stained with hematoxylin–eosin and periodic acid Schiff for light microscopy observation. The other was used for functional amylase secretion measurements, basal and nerve-mediated [with electrical field stimulation (EFS)] and secretagogue-evoked secretion [acetylcholine (ACh) 10^{-5} M] with established fluorimetric techniques.

RESULTS: All the rats exposed to LFN showed marked morphological lesions including vacuolar degeneration, hyalinization, cell death and blood vessels wall focal thickening. No morphological changes were detected in the control group. The functional results presented significantly diminished basal, EFS and ACh-evoked amylase secretion when compared with the control group.

CONCLUSION: These results suggest that the parotid gland is a target for LFN-induced lesions with an impairment of its function.

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Keywords: low-frequency noise; parotid gland; vibroacoustic disease; histology; amylase secretion

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Introduction

Low-frequency noise (LFN) with low spectral energy, bandwidth up to 500 Hz and amplitude of 80–110 dB, is present in a diversity of environments ranging from professional or residential to leisure (Alves-Pereira, 1999). The presence of LFN is normally not assessed during routine noise evaluations but LFN sources are present in working environments such as onboard ships, oil rigs, and space or in the textile industry, and in urban and suburban environments such as near high-volume roads, highways, subways or bus stations. In some leisure environments such as discotheques, LFN is also a constant presence.

The term vibroacoustic disease (VAD) was proposed to describe the spectrum of systemic alterations produced in humans after long-term exposure (>10 years) to LFN. VAD is characterized by a progressive fibrosis of tissues, via extracellular matrix proliferation, edema and cellular death as well as epithelial alterations in the absence of inflammatory process (Alves-Pereira, 1999; Castelo Branco, 1999a). Moreover, fibrosis is a constant feature found in LFN-exposed animals and in humans (Bovenzi *et al*, 1995; Alves-Pereira, 1999; Castelo Branco, 1999a,b; de Sousa Pereira *et al*, 1999; Oliveira *et al*, 2002a).

Several systems, such as the cardiovascular, have been described to be affected by the noxious effects of LFN. Thickening of the cardiac valves and the pericardium has been documented echographically (Castelo Branco *et al*, 1999a). The blood vessels presented thickening of the intimal layer and disruption of the internal elastic lamina (Martins dos Santos *et al*, 2002). Lymphatics also presented thickening of the walls (Grande *et al*, 2001; Martins dos Santos *et al*, 2004). In the respiratory system, trachea brush cells and ciliated cells were found altered (de Sousa Pereira *et al*, 1999; Oliveira *et al*, 2002a,b). The pulmonary parenchyma showed marked thickening caused by fibrosis (Castelo Branco *et al*, 2004). The digestive (da Fonseca *et al*, 2005), urinary, nervous, or immune system can also be affected (Castelo Branco *et al*, 1999b); in the digestive system,

ultrastructural studies have shown cellular degeneration and death; and at the cellular level DNA alterations are also documented.

The underlying mechanism of the deleterious effects of LFN is not yet understood. It is known that in low-frequency bandwidths, noise is associated with vibration through resonance which may have a significant role in the development of the reported alterations. These findings suggest that the parotid glands are a potential target for LFN-induced lesions.

To our knowledge, no previous studies were performed on this gland. Therefore, the goal of this study was to evaluate the effects of LFN on the morphology and function of the parotid gland of Wistar rats.

Materials and methods

Animals

All animal experiments were performed on isolated parotid glands taken from 20 adult (250–350 g) CD strain Wistar rats, male and female, in equal number, age ranging between 8 and 12 months.

The animals used in this study were randomly divided into two groups. Ten rats were assigned to group 1 (LFN-subjected group) and submitted to 2184 h (13 weeks) of continuous LNF while the other half were assigned to group 2 (control group). These were kept in similar laboratory conditions but in silence. The rats were kept in cages with two or three animals per cage, setting no limits on their movements, and exposing them to cycles of 12 h light/dark. All animals were fed standard rat food, had unrestrained access to water, and were treated in accordance with the EU directive on Animal Protection for Experimental and Scientific Purposes (86/609/CE) and also according to the relevant Portuguese laws.

The animals were killed by a blow to the head followed by cervical dislocation. An incision was made in the upper part of the neck, and the parotid glands were surgically removed. One of the glands was used for histopathologic observation while the other was used for functional amylase secretion measurements.

Low-frequency noise exposure protocol

In an isolated compartment measuring $217 \times 211 \times 195$ cm the cages were placed in front of a noise generator, consisting of a subwoofer Magnat xtc 1200 (Magnat, Pulheim, Germany). A computer Compaq (Pentium 133 MHz) (Compaq, Palo Alto, USA) reproduced the noise previously recorded. The sound signal was amplified with a QSC amplifier and frequency-filtered (QSC Audio Products CA Inc., Costa Mesa, CA USA), creating an acoustic environment rich in low-frequency components. The noise produced was measured with a digital spectral analyzer. The spectral analysis was similar in all the compartments. Levels were above 90 dB in the frequencies ranging from 50 to 500 Hz.

Materials

All the chemicals were purchased from Sigma (Madrid, Spain) and were of the highest purity available.

Morphologic study – experimental protocol

The parotid glands were removed and kept in 10% buffered formalin for light microscopy (LM). Sections were stained with hematoxylin–eosin and periodic acid Schiff (PAS) by standard methods. The investigator who examined the morphologic alterations in the salivary glands was blinded to the treatment groups.

Functional study – experimental protocol

Amylase measurements

The parotid glands were quickly removed and immersed into a modified Krebs-Henseleit (KH) solution comprising (mM): NaCl, 103; KCl, 4.76; $CaCl_2$, 2.56; $MgCl_2$, 1.1; $NaHCO_3$, 25.0; NaH_2PO_4, 1.15; D-glucose, 1.8; sodium pyruvate, 4.9; sodium fumarate, 2.7; and sodium glutamate, 4.9. The solution was kept at pH 7.4 while being continuously gassed with a mixture of 95% O_2:5% CO_2 and maintained at 37°C.

Measurements of amylase output were obtained by established methods (Rinderknecht and Marbach, 1970). Briefly, the parotid glands were cut into small segments (10–25 mg) and a total weight of about 175–200 mg was placed in a custom-made Perspex flow chamber (vol = 2 ml) and superfused with oxygenated KH solution at 37°C at a flow rate of 1.8 ml min^{-1}. The effluent from the chamber passed directly to a peristaltic pump-driven, online automated fluorimetric assay for the continuous measurement of amylase output. Amylase output data were collected directly into a computer and intensity expressed via a winlab software. Subsequent conversion of fluorescence intensity was performed to express the results as units of amylase per ml per 100 mg of tissue. One parotid gland provided enough tissue which allowed a total of three experiments to be performed. After each experiment, the tissue was removed from the chamber, blotted dry and weighed. α-Amylase [30 U ml^{-1} (Sigma type II-A)] was used as a standard to calibrate the assay, both at the beginning and at the end of each experiment. Basal amylase output was obtained after allowing the tissue to stabilize for 40 min.

For the study of nerve-mediated secretion, a special Perspex tissue chamber containing silver wire electrodes for electrical field stimulation (EFS) was employed. The parameters of EFS were: amplitude, 50 V; frequency, 5, 10, or 20 Hz; pulse duration, 1 ms. In another set of experiments secretagogue stimulations with acetylcholine (ACh) 10^{-5} M were evoked by adding ACh directly to the superfusing chamber. Either EFS or ACh stimulation was always applied for a 10-min period. Each tissue sample was stimulated once (upon tissue stabilization) allowing for a total number of 15 amylase secretion measurements per stimulating agent.

Statistical data analysis

Data were recorded as amylase output or as mean ± standard error of the mean (s.e.m.) of amylase basal values or amylase above basal values as appropriated. Kolmogorov–Smirnov tests were conducted to verify normal distribution within each group. Data were found to be not normally distributed. Therefore,

controls and test values were compared using nonparametric tests (Mann–Whitney) (SPSS for Windows version 13.0). $P < 0.05$ was considered as significant.

Results

Morphologic

The general aspect of the glandular parenchyma of the LFN-exposed animals showed marked alterations with distortion of its global architecture in all the studied sections. We observed fibrosis of the intralobular perivascular and peritubular connective tissue that had a whirled aspect. The interlobular and perilobular connective tissue also presented marked fibrosis with hyalinization.

As seen in Figure 1 the stroma showed acinar anarchy and signs of cellular death, degenerative vacuolar changes of acinar cells with irregular shape and size that spared the tubular cells of the excretory system. These vacuoles reacted negatively to PAS. There were no inflammatory cells in the stroma.

We found marked alterations in blood vessels that consisted of focal thickening of the walls which in some cases protruded to the lumen as thrombotic-like formations (Figure 2). All control sections were normal.

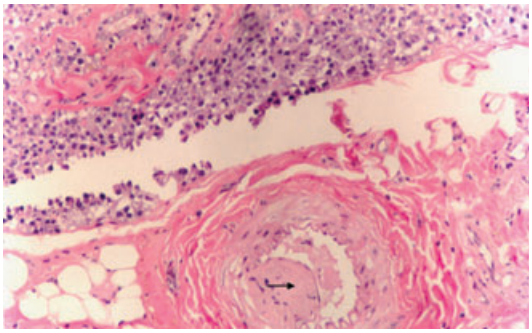

Figure 1 Parotid gland (HE, ×400) of a low-frequency noise-exposed animal with degenerative vacuolar changes of acinar cells (↑), intralobular fibrosis (↑↑), and a thrombotic-like lesion of a small vessel (↑↑↑)

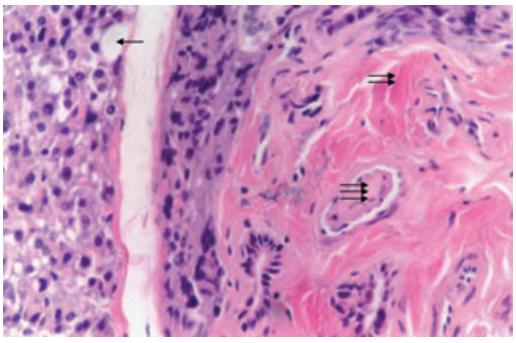

Figure 2 Parotid gland (HE, ×200) of a low-frequency noise-exposed animal. Vascular lesion of a medium size artery protruding to the lumen (↑)

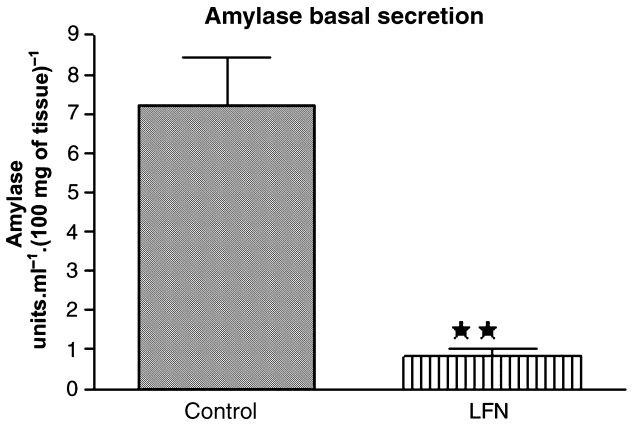

Figure 3 Histograms showing basal amylase secretion from isolated parotid gland segments. Each point is mean ± s.e.m., $n = 30$. Basal amylase output was obtained after allowing tissue to stabilize for 40 min. Note that amylase secretion is significantly decreased ($P < 0.01$, Student's *t*-test) for low-frequency noise-submitted animals compared with the matched controls

Functional

Amylase measurements

Figure 3 shows basal amylase output from superfused parotid segments of rats submitted to LFN for 13 weeks compared with the controls. The results showed that in rats that were submitted to LFN the basal amylase output was significantly inhibited ($P < 0.05$) when compared with the matched controls. Results report to a total of 30 experiments for each group.

Figures 4 and 5 show the effect of EFS 10 Hz 50 V and ACh 10^{-5} on amylase output of control and LFN submitted rat parotid glands, respectively. Original chart recordings of the responses for EFS and ACh are shown in Figures 4a and 5a and the mean (±s.e.m.) amylase output above basal level for EFS and ACh are shown in Figures 4b and 5b, respectively. The results show that in rats submitted to LFN for 13 weeks, EFS and ACh-evoked amylase output above basal levels were extremely significantly diminished ($P < 0.01$) when compared with the control groups. Results report to a total of 15 experiments for each group (ACh and EFS).

Discussion

In our study the animals were exposed continuously to LFN to simulate environments rich in low-frequency components. Fibrosis and degenerative vacuolar changes were the major findings in LM observations of the LFN-exposed animals. There seems to be a destructive process of the glandular parenchyma which is consistent with the functional results. The content of the vacuoles reacted negatively to PAS, suggesting that some substance, which disappears with sample processing, probably fat, could be present. This hypothesis could not be confirmed in this study as the method used for tissue fixation was not appropriate for lipid studies. Our findings are partially similar to those found in rats subjected to an ethanolic diet for long periods (Tirapelli *et al*, 2001; Faustino and Stipp, 2003), in

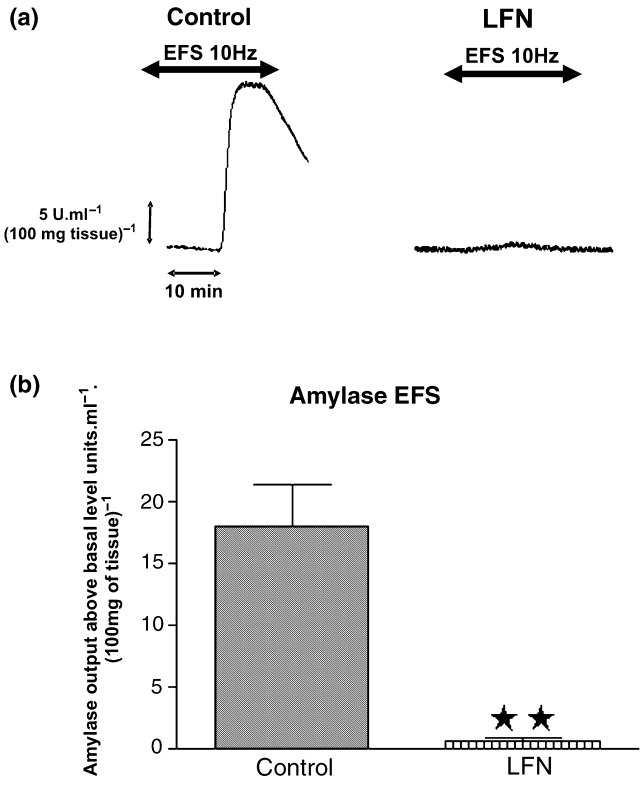

Figure 4 (**a**) Original chart recordings showing the effect of electrical field stimulation (EFS) at 10 Hz (amplitude of 50 V and 1 ms pulse width) on amylase output from control and low-frequency noise (LFN)-submitted superfused rat parotid segments. Traces are typical of 15 experiments. (**b**) Histograms showing the mean (±s.e.m.) amylase output above basal level for EFS at 10 Hz of control and LFN-submitted superfused rat parotid segments, $n = 15$ and taken from five animals. Note that amylase secretion is significantly decreased ($P < 0.01$, Student's *t*-test) for LFN-submitted animals compared with the matched controls

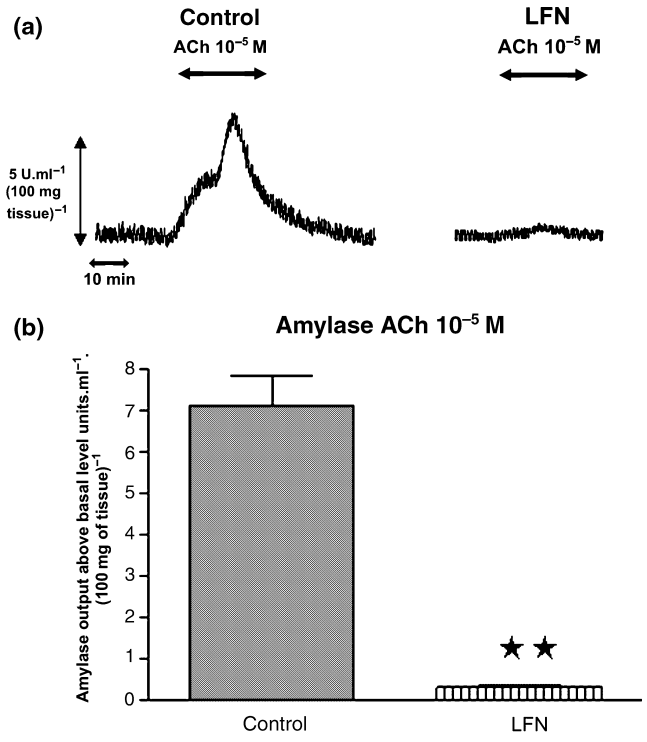

Figure 5 (**a**) Original chart recordings showing the effect of 10^{-5} M acetylcholine (ACh) on amylase output from control and low-frequency noise (LFN)-submitted superfused rat parotid segments. Traces are typical of 15 experiments. Traces are typical of 15 such experiments taken from five animals. (**b**) Histograms showing the mean (±s.e.m.) amylase output above basal level for 10^{-5} M ACh stimulation. Note that amylase secretion is significantly decreased ($P < 0.01$, Student's *t*-test) for LFN-submitted animals compared with the matched controls

which 'fat degenerescency' and acinar atrophy leading to diminished salivary gland parenchyma and secreted flow were associated with ductal changes. However, after 13 weeks of LFN exposure, ductal changes were not present in the observed specimens. The presence of cytoplasmic vacuoles and fibrotic areas are common morphological findings in irradiated animals (Coppes *et al*, 2001), suggesting that these signs are similar in the processes leading to a decreased or non-functioning parotid parenchyma. Moreover, vacuolization, and subsequent diminishing of functional parenchyma, is also found in animals maintained on a liquid diet leading to gland atrophy (Coelho Leal *et al*, 2003). Liquid diet feeding is another situation which has been shown to lead to acinar atrophy and diminished salivation. Mastication is known to be a powerful stimulus for salivary secretion acting through receptors which are present in mastication muscles, periodontal ligament or mucosa via the trigeminal and superior salivary nuclei, eliciting an increase in parasympathetic secretomotor discharge (Edgar, 1992). Animal studies using the rat model have shown that rats fed on a liquid diet present diminished salivary stimulated flow (Scott *et al*, 1990). Moreover, it has also been shown that diminished salivation resulting from a liquid diet is linked to the presence of acinar atrophy (Scott and Gunn, 1991) and to a decrease in muscarinic and β_1 adrenergic receptor acinar density (Johnson and Cardenas, 1993). More recently, vacuolization and subsequent diminishing of functional parenchyma were also found in animals maintained on a liquid diet leading to gland atrophy (Coelho Leal *et al*, 2003). Although rats used in this study were fed on hard chow, the vacuolization of acinar cells found in LFN-submitted animals shares some similarities with others described above, of different causes, and could also correspond to a sign of gland atrophy. Thus the vacuolization we found may be a sign of gland atrophy.

In previous animal studies our group found severe alterations in lymphatic vessels and in small and medium-sized arteries of LFN-exposed animals (Grande *et al*, 2001; Martins dos Santos *et al*, 2002, 2004). These alterations consisted of focal thickening of the intima, disruption of the internal elastic lamina and proliferation of smooth muscle cells in the aorta and femoral artery, and disruption of the valvular apparatus of the lymphatics. The vascular alterations found in the interstice of the gland are very similar to the ones that occur in a process known as necrotizing sialometaplasia, a condition that often appears spontaneously or associated with surgical manipulation of the parotid gland

(Imbery and Edwards, 1996). In necrotizing sialometaplasia, blockage of local blood supply causes 'infarction' of the glandular lobes with maintenance of the ductal system. The vascular lesions could be responsible for the strong hyalinization of the connective tissue and diminishment of functional parenchyma observed in this study.

Functional results have demonstrated that basal amylase secretion is significantly diminished in rats submitted to LFN compared with the controls, suggesting that this condition induces functional modifications in the resting gland.

The autonomic sympathetic and parasympathetic nerves release the neurotransmitters noradrenaline (NA) and ACh, respectively. Both ACh and NA trigger mainly a sequence of cellular signal transduction events resulting in intracellular cascades to generate second messengers such as calcium (Ca^{2+}) and adenosine 3,5 cyclic monophosphate (cyclic AMP) which in turn activate ion transport pathways, water, and protein secretion a process known as stimulus secretion coupling (Baum, 1987; Petersen and Gallacher, 1998). Therefore, it seemed logical to investigate the effects of LFN on nerve-mediated stimulation of amylase secretion in the rat parotid glands. The technique of EFS has been widely used as a physiologic tool for activating intrinsic nerves in a number of secretory tissues to study the effect of the release of endogenous neurotransmitters and their secretory effects (Mata *et al*, 2002; Yago *et al*, 2002).

The results of this study have shown that electrical stimulation of intrinsic secretory nerves can result in marked increases in amylase output in control rat parotid segments. This is in agreement with the previous findings by others in the literature (Yago *et al*, 2002). However, the results demonstrate that the magnitude of the EFS-induced amylase release is significantly reduced in the parotids of the rats exposed to noise and vibration indicating an LFN-induced functional impairment of the gland. The diminished secretory capacity verified in the EFS experiments could derive from LFN-induced nervous tissue damage, from acinar cell damage or from both. As the morphological results showed profound LFN-induced parenchyma lesions involving acinar cells, it seemed logical to study direct cellular stimulation in order to ascertain whether the acinar cells maintained their secretory capacity. The addition of cellular secretory agonists such as ACh to the super-fusing medium has been used before by others and is widely accepted as a good method for studying direct stimulation of acinar cells (Mata *et al*, 2002). Sympathetic activation is known to be the main stimulus for protein secretion, while parasympathetic stimulation controls mainly fluid secretion. ACh which is released from the parasympathetic nerves acts through the intracellular generation of IP_3 and diacylglcerol (DAG). IP_3 triggers an intracellular calcium rise which is responsible for fluid and ion secretion. In addition, DAG activates protein kinase C which is responsible for ACh-evoked protein secretion. Therefore, a diminishment in ACh-evoked amylase secretion could indicate a cellular impairment which would also affect fluid secretion. ACh-evoked amylase secretion from the parotid segments of control rats has shown an elevation above the basal levels with a smaller magnitude when compared with the EFS-evoked secretion, which is in agreement with previous studies (Yago *et al*, 2002). However, ACh-evoked amylase secretion above basal levels in rats exposed to LFN was found to be significantly diminished when compared with the controls, indicating the presence of impaired acinar cell function consistent with the diminishment of gland functional parenchyma observed in LM preparations. Future studies should try to ascertain which steps of the signaling pathway may be altered upon exposure to LFN. Moreover, these results could further indicate that LFN exposure may lead to salivary gland dysfunction or xerostomia. This could be of clinical interest and should be investigated in future studies. Salivary flow changes upon exposure to LFN which were not investigated in this preliminary study should be performed.

Presently, there is no knowledge to discern whether the lesions observed result from a direct acoustic-induced trauma or from some other pathophysiologic process secondary to the noise. In this particular case, we do not know whether the lesions are caused by the severe vascular compromise that we have clearly observed in the gland. Taken together, the results of this study demonstrate that the parotid gland can be affected by LFN.

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