

ARTIGO ORIGINAL/ORIGINAL ARTICLE

O epitélio respiratório em ratos Wistar nascidos em ruído de baixa frequência e expostos a ruído adicional

Respiratory epithelia in Wistar rats born in low frequency noise plus varying amounts of additional exposure

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RESUMO

A exposição ao ruído de baixa frequência (RBF) (≤ 500 Hz, incluindo os infra-sons) provoca lesões no epitélio do aparelho respiratório. Em ratos cuja gestação ocorreu sob o efeito de RBF, e que subsequentemente passaram um ano em

ABSTRACT

Earlier studies of Wistar rat respiratory epithelia exposed to low frequency noise (LFN) (≤ 500 Hz, including infrasound) showed that LFN effects trauma on the respiratory tract. In rats gestated and born in LFN environments, trauma was still

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Recebido para publicação/Received for publication: 03.09.22

Aceite para publicação/Accepted for publication: 03.10.10

silêncio, observaram-se lesões estabilizadas e definitivas do epitélio respiratório. Neste estudo, investigam-se as lesões que se observam em ratos nascidos em RBF e expostos a RBF adicional. Ratos nascidos em RBF foram posteriormente expostos a RBF adicional antes de serem sacrificados: 145 h (Grupo A), 235 h (Grupo B), 2213 h (Grupo C), 2438 h (Grupo D), 4399 h (Grupo E) e 5304 h (Grupo F). Todos os animais foram tratados de acordo com a norma aplicável (86/609/CE). Fragmentos do epitélio respiratório foram processados para microscopia óptica e electrónica. No grupo A, as microvilosidades das células em escova (CE) juntam-se, deixando de ter a distribuição uniforme observada nos ratos de controlo; no grupo B as microvilosidades das CE aglomeram-se e, nos restantes grupos, apresentam-se fundidas. As rosetas (anéis de células secretoras centradas numa CE) são visíveis nos grupos A-D e tornam-se difíceis de identificar nos grupos E, F. A quantidade de cílios “ceifados” aumenta com o tempo de exposição, bem como as imagens de desdiferenciação celular. O RBF produz um efeito nefasto sobre o epitélio respiratório destes ratos.

REV PORT PNEUMOL 2003; IX (6): 481-492

Palavras-chave: ruído de baixa frequência, doenças do colagénio, célula em escova, cílios, microvilosidade, desdiferenciação, doença vibroacústica, metaplasia, displasia, carcinoma pavimentocelular.

evident after spending 1 year in silence. This report studies Wistar rats gestated and born in a LFN environment and exposed to additional LFN. Wistar rats were gestated and born while exposed to LFN. After birth, the following groups were exposed to LFN for an additional 145 hrs (Group A), 235 hrs (Group B), 2213 hrs (Group C), 2438 hrs (Group D), 4399 hrs (Group E), and 5304 hrs (Group F). All animals were treated in accordance with 86/609/EC. Respiratory epithelial fragments were prepared for light and scanning/transmission electron microscopy. Group A brush cell (BC) microvilli tended to group together; in Group B they were clearly clustered together, and in Groups C-F they became fused. Rosetta structures (rings of secretory cells centered on a BC) were visible in Groups A-D and difficult to identify in Groups E,F. The amount of sheared cilia increased with exposure time, as did the images of cellular de-differentiation. LFN exposure induces severe trauma on the respiratory epithelial cells in these rats.

REV PORT PNEUMOL 2003; IX (6): 481-492

Key-words: low frequency noise, brush cell, collagen diseases, ciliated cell, microvilli, cilia, de-differentiation, vibroacoustic disease, metaplasia, displasia, squamous-cell carcinoma.

INTRODUCTION

Vibroacoustic disease (VAD) is a whole-body pathology caused by long-term exposure (years) to low frequency noise (LFN) (≤ 500 Hz, including infrasound)¹⁻³. Initially identified in aircraft technicians^{1,2,4}, it has since been confirmed in a population of commercial airline

pilots and crewmembers⁵, as well as in a population of islanders exposed to environmental LFN generated by military training exercises⁶.

Within the context of LFN-rich environments, an informal survey of a textile factory (today deactivated) was conducted in 1987. An unusual amount of teratogenic malformations were observed in the children of female textile workers.

Concurrently, the involvement of the respiratory system in LFN-induced pathology had intrigued this team since these studies began in 1980. Consequently, the use of Wistar rats as animal models to study the non-auditory pathology caused by exposure to LFN began in 1992.

These animal model studies have provided a wealth of information on how LFN targets the respiratory system⁷. Much of the data obtained to date has greatly contributed to explain the many respiratory signs and symptoms observed in LFN-exposed workers^{2,8,9}. Third generation rats born in an LFN environment were observed to have teratogenic malformations (Fig.1).

Subsequently, a study was conducted on the respiratory epithelia of rats gestated and born in LFN, and then kept in silence for one year. It showed that LFN-induced lesions, such as depleted ciliary fields and structural abnormalities in brush cells, lingered well after the acoustic

stressor had ceased to be present⁷. Since silence is not such an abundant commodity, especially in urban and suburban settings, the model would more closely correspond to real life situations if it included LFN exposure during the post-birth years, instead of one year in continuous silence.

In this report, a progression of LFN-induced lesions is investigated in the respiratory epithelia of Wistar rats gestated and born in a LFN environment, and additionally exposed to varying amounts of LFN.

METHODS

Noise exposure

A sound signal was generated by an analog noise generator, amplified and frequency filtered. Fig. 2 shows the overall linear and A-weighted

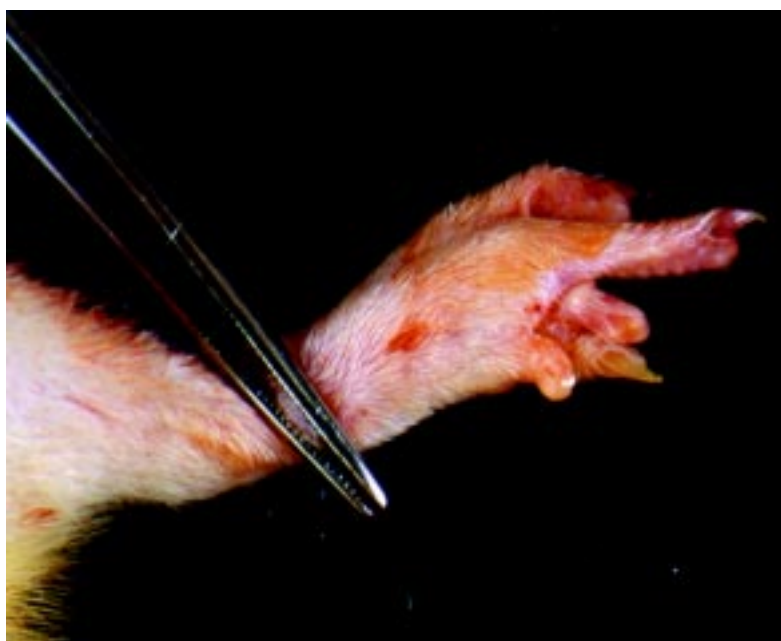


Fig. 1 — Limb of a third generation rat born within a LFN environment with evident gross malformations that include loss of segments.

Animal Model Low Frequency Noise Exposure

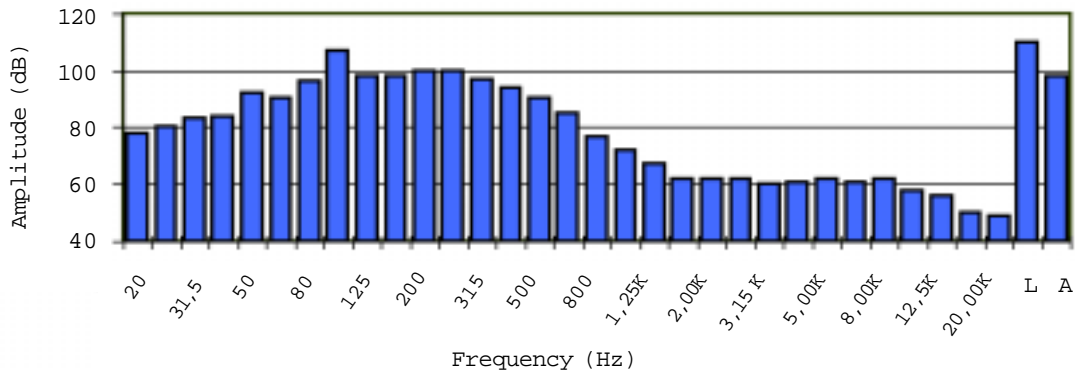


Fig. 2 — Frequency distribution of rat LFN exposure. Average levels: 109 dB_{L_{im}} (L), 98 dBA (A).

noise levels, as well as the spectral analysis of the excitation signal collected at the position near the rat test group inside the chamber. This noise was analyzed by a digital real time analyzer (B&K 2144, Denmark). In this experiment the sound energy was highly concentrated in the lower frequency bands due to the influence of the low-pass filter. In the frequency bands ranging from 50 Hz to 500 Hz the noise levels exceeded 90dB. The overall levels were registered above 109dB, with the A-weighted levels being around 98dB (A)

Animals

Wistar rats were gestated and born in an occupationally-simulated LFN environment, consisting of the following exposure schedule: 8 hours/day, 5 days/week, weekends in silence.

Following birth, groups of five rats were exposed to additional LFN as follows: Group A – 145 hrs; Group B - 235 hrs; Group C – 2213 hrs; Group D – 2438 hrs; Group E – 4399 hrs; and Group F – 5304 hrs.

All post-birth exposed rats were sacrificed after spending one week in silence to attenuate the effects of cellular tumefaction⁷. Ten age-matched rats gestated, born, and living in silence were used as controls. Rodents were obtained from a local breeder (Gulbenkian Institute of Science, Oeiras, Portugal), had unrestrained access to water, and were treated in accordance with the European Commission on Animal Protection for Experimental and Scientific Purposes (86/609/CE).

Fragments from the respiratory system were excised and prepared for light microscopy, and scanning (SEM) and transmission (TEM) electron microscopy.

Microscopy

The animals were sacrificed by a lethal intravenous injection of sodium-pentobarbital (40mg · kg⁻¹ BW) and the trachea was divided in two, along the saggital line. Specimens for light microscopy were formalin-fixed, paraffin-embed-

ded, and stained with hematoxylin-eosin, and fucsin-rhesorcin.

Specimens for electron microscopy were placed in a solution of 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 and then washed with several changes of 5% sucrose in 0.1 M phosphate buffer, pH 7.2, for ultrastructural studies.

Specimens for SEM were dehydrated, critical point-dried and coated with gold-palladium. Examination with the electron microscope (JEOL JSM-35C, Japan) was performed at an accelerating voltage of 15 kV.

For TEM, samples were fixed at room temperature in an aldehyde mixture consisting of 4% paraformaldehyde, 1.25% glutaraldehyde, and 10mM CaCl₂ in 0.05 M cacodylate buffer, and pH 7.2. Specimens were washed in buffer, and postfixed in a ferricyanide-reduced osmium solution made up of 1% potassium ferricyanide and 1% osmium tetroxide in distilled water, dehydrated through a graded ethanol series, and embedded in Epon. The samples were sectioned in an ultramicrotome (LKB, Sweden) and the thin sections stained with uranyl acetate and lead citrate. Preparations were then examined with electron microscopy (JEOL 100C, Japan).

RESULTS

Group A (145 hours post-birth LFN exposure)

Cilia population was exuberant, although some cilia appeared shaggy, unlike in controls where fields of cilia could be observed in metachrony coordination. Secretory cell (SC) microvilli were long and of different sizes, indicating different stages of life cycles, as in controls. Rosetta-shaped structures were visible, consisting of a ring of SC centered on a brush cell (BC) (Fig. 3).

Intercellular junctions of the rosetta were demarked by valleys due to the presence of re-

sidual cellular tumefaction. BC microvilli were individually visible, and uniformly distributed over the apical surface of the BC in contact with the airway. In Figure 3, two pore-like black spots are visible. These are not artifacts and were seen in numerous micrographs. The function of these pores is unknown.

Group B (235 hours post-birth LFN exposure)

Cilliary population was visibly shaggy, and sheared cilia were frequently observed. SC microvilli were shorter than in controls, and appeared stunted (Fig. 4). BC microvilli grouped together, forming regular clusters of microvilli, and lost the uniform distribution seen in controls.

Intercellular junctions were clearly identifiable, although the deep valley borders were not as evident as in Group A. The rosetta structures became more prominent, and were always clearly visible, although in some, BC seemed to be missing and appeared to have begun a necrotic process.

Group C (2213 hours post-birth LFN exposure)

The amount of cilia was visibly reduced, and existing cilia were shaggy and sheared. BC microvilli were observed in clusters, unlike in controls or in Group A. Individual microvilli ceased to be identifiable. SC microvilli remained overall short and stunted. The shape of the rosetta structure lost its regularity, particularly because SC lost their uniform shape.

Intercellular junctions were thick and prominent, clearly demarking the rosetta structures. The cellular landscapes observed in this group were often suggestive of a de-differentiation process.

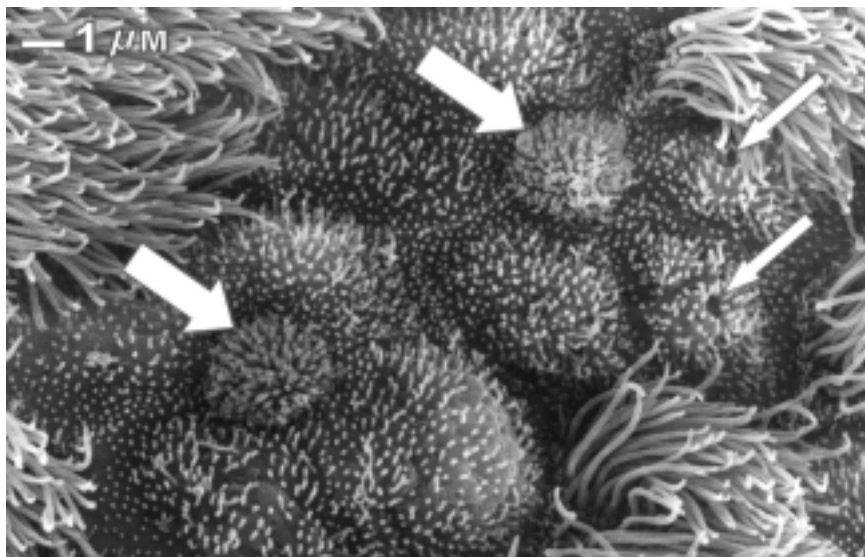


Fig. 3 — SEM of Group A (145 hours) rat tracheal epithelium. Cilia are discretely shaggy. Brush cells (BC) (large arrows) are visible at the center of a ring of secretory cells (SC), forming a rosetta-shaped structure. BC microvilli are individually visible. SC microvilli are exuberant and at different lengths. Due to residual cellular tumefaction, intercellular junctions are marked by deep valleys. The two holes (small arrows) are not artifacts (see text), and were observed in other micrographs of respiratory epithelia.

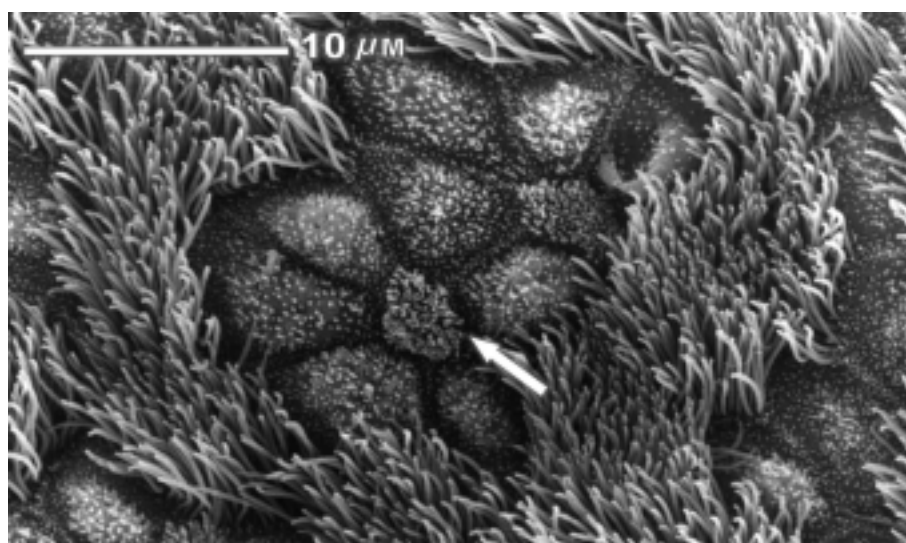


Fig. 4 — SEM of Group B (235 hours) rat tracheal epithelium. Cilia are discretely shaggy. The rosetta structure is readily identifiable, centered on the BC (arrow). BC microvilli are grouped together, losing the uniform distribution seen in non-exposed controls. SC microvilli are short and stubby. Intercellular junctions are clearly visible, although the deep valley border is less evident than in Group A.

Group D (2438 hours post-birth LFN exposure)

In TEM, the relative position of the BC surrounded by SC, forming the rosetta structure, was confirmed. In Groups A and B, BC shape was cylindrical and grew pyriform with age, similar to controls. In Groups C-F, most BC were larger, with a pyriform-like shape, and many contained vacuoles (Fig. 5). In Group D, BC containing large vacuoles were more frequent, and some BC become large empty membranes (Fig. 6). The subepithelial layer consisted of hyperplastic collagen bundles, some exhibiting a degenerative pattern (Figs. 5, 6).

Multivesicular bodies appeared inside the BC in localized areas, as in controls, but were larger

and more numerous in the exposed specimens. BC always appeared close to fiber bundles, and possessed many interdigitations to neighbouring SC. Ciliary axoneme internal structure was intact, but the number and distribution of basal bodies was reduced and uneven. The number of organelles was decreased and cilia shape lost uniformity. Budding vesicles from cilia and BC microvilli were observed in all micrographs. These vesicles were reduced in the exposed, as compared to controls. In controls, SC vesicles appeared compact and organized as they moved toward the airway. However in the exposed, these vesicles were disorganized, with large spaces in between, and their electronic density was irregularly increased.

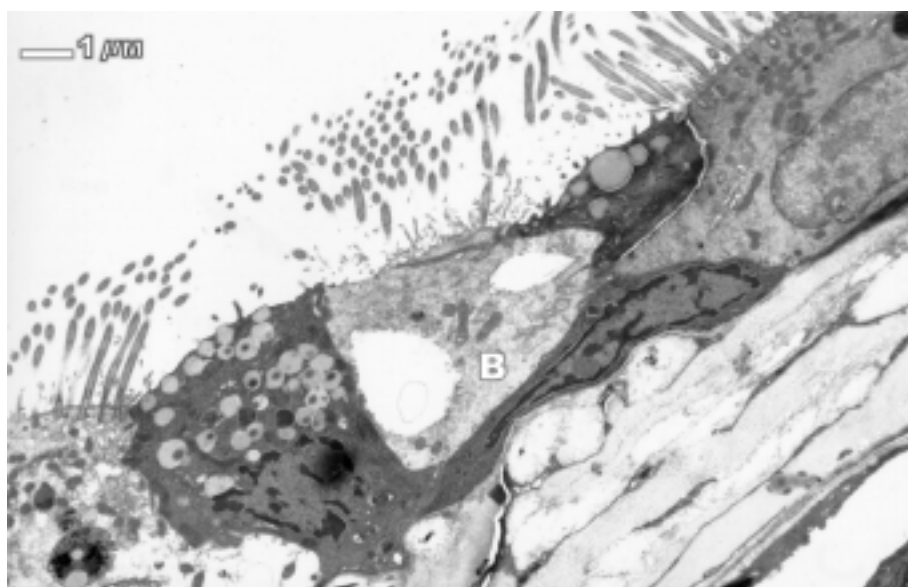


Fig. 5 — TEM of Group D (2438 hours) rat tracheal epithelium. A BC (B) containing two vacuoles and sided by two SC. Secretory vesicles emanating from the SC are of different sizes and are no longer evenly distributed. Below the BC, a thick layer of collagen is very evident.

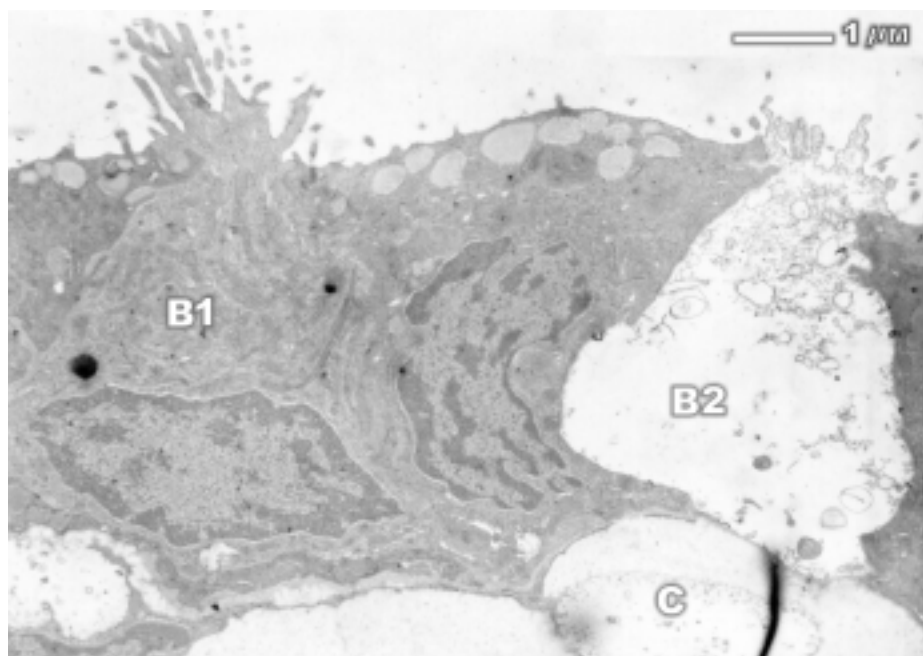


Fig. 6 — TEM of Group D (2438 hours) rat tracheal epithelium. A young BC (B1) and an older BC (B2) with a SC in between. Microvilli of both BC protrude into the lumen. A thick layer of collagen (C) is visible below B2.

Group E (4399 hours post-birth LFN exposure)

Bald ciliated cells were frequently observed. The amount of cilia was further reduced when compared to the other groups, and the remaining cilia were shaggy and sheared (Fig. 7). SC microvilli continued short and stubby, unlike controls. Images of de-differentiating cells were frequently identified. BC microvilli became fused, and could be seen spreading outward from the center.

Group F (5304 hours post-birth LFN exposure)

Sheared cilia appeared very frequently, and short, shaggy strands of cilia were often visible.

SC microvilli remained stunted. Rosetta structures were not easily identifiable, but their location could be inferred by the relative positions of the SC (Fig. 8). Cellular de-differentiation appearances were frequently observed. Sunken BC were commonly seen. BC microvilli are always fused with central indentations.

DISCUSSION

The progression of trauma observed in the rat respiratory epithelia after LFN exposure unequivocally demonstrates that this acoustic stressor is an agent of disease. The function of the respiratory BC is, as yet, unknown^{10,11}. Afferent innervation and absorption features have been associated with the BC, but most studies have focused on digestive tract epithelia^{10,12}. The rea-

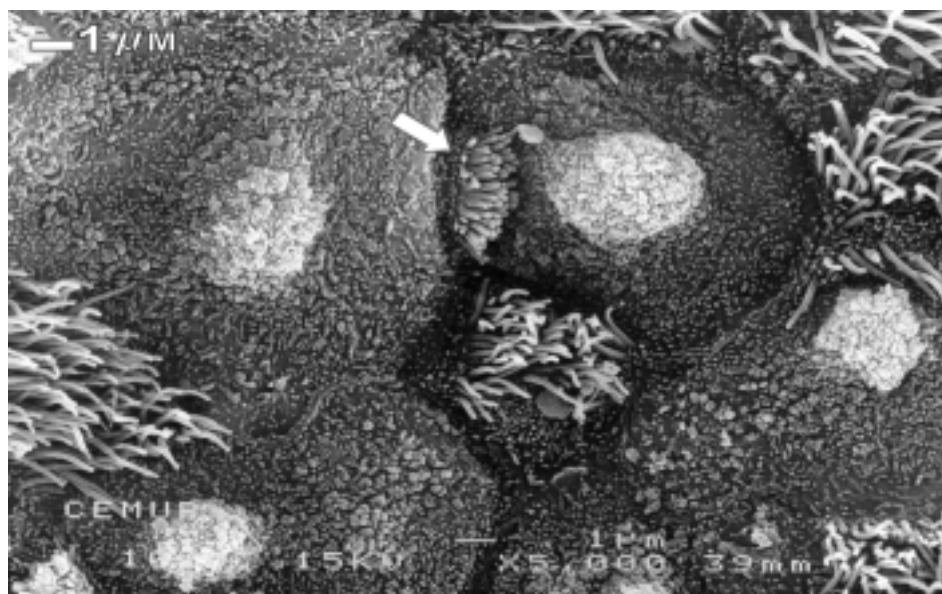


Fig. 7 — TEM of Group E (4399 hours) rat tracheal epithelium. BC (arrow) with SC on the right-hand side, as well as the ciliated cell below, are sunken. The landscape exhibits cellular disorganization.

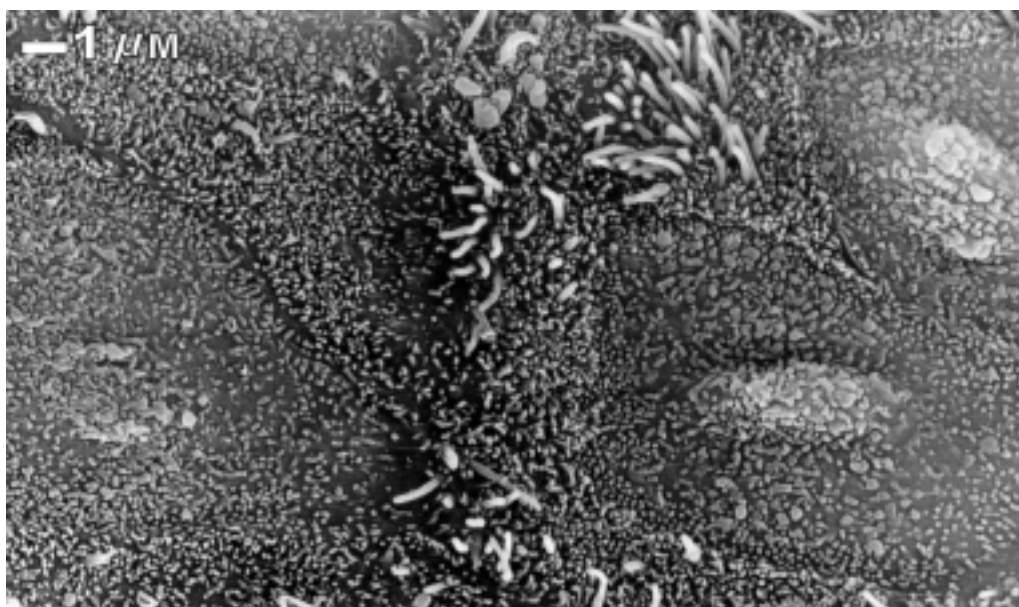


Fig. 8 — TEM of Group F (5304 hours) rat tracheal epithelium. Devastated SC and ciliated cells. No BC are visible and cellular organization is erratic.

son why BC and SC microvilli are so vulnerable to LFN stress is unknown, as are the mechanisms through which BC microvilli fuse. Nor is it understood why they fuse. Clearly the behavior of the BC under LFN exposure is intriguing and future biochemical studies might yield important information.

After 2438 hours of occupationally-simulated LFN exposure, appearances of cellular de-differentiation were identifiable in Groups D-F (Fig. 6). The genotoxic effect of LFN on human and animal models has already been the object of several studies, where all LFN-exposed populations exhibited a statistically significant increase in the frequency of sister chromatid exchanges^{13,14}. In LFN-exposed workers, the only type of respiratory tract neoplasms that have been observed are squamous-cell carcinomas⁹. In a previous study of 236 VAD patients, 28 individuals developed malignancies, 5 of whom had multiple tumors². Of these 28 cases, 5 were squamous cell carcinomas of the lung, 2 in non-smokers. Since then, other cases of squamous cell carcinomas of the lung have been identified in LFN-exposed professionals⁹. The data presented herein warrant a close look at the relationship between LFN exposure and neoplasms of the respiratory system, particularly squamous cell carcinomas.

Ciliary behavior also deserves an in-depth analysis. The internal ciliary structure is intact, as confirmed by TEM micrographs. But as exposure time increases, cilia appear shaggy and sheared, and the basal bodies appear to be in involution. In Fig. 8, a ciliated cell with sheared cilia is clearly visible in the vicinity of other ciliated cells with longer, but shaggy, cilia. Both mechanical and biochemical events may be responsible for this pattern of trauma.

Budding vesicles from cilia and BC microvilli were seen in controls and, to a lesser extent, in exposed animals⁷. Morphometric studies of this particular feature are currently underway. The notion of cilia as a secretory structure was un-

known to these authors, and, to the authors' knowledge, was only documented in one other location of the medical literature¹⁵. The implications of this observation are still under study.

The rosetta structure has previously been identified in LFN exposed Wistar rats⁷. In controls, rosetta structures are more difficult to identify because fields of well developed and exuberant cilia cover many of the BC and SC that form the rosettas. But in TEM, both in exposed and controls, the relative position between SC and BC is confirmed, with many interdigitations emanating from the BC body into the surrounding SC⁷. Multivesicular bodies were observed within well defined areas of the BC in controls and exposed, although in the latter they appeared increased in size and number⁷. These multivesicular bodies are the object of ongoing studies that have already determined their neuropeptide nature^{16,17}. The reason why these epithelial cells are grouped in rosetta structures is unknown, and to the authors' knowledge, this structure has not been identified elsewhere in the medical literature.

The hyperplastic appearance of collagen bundles is in accordance with the previously observed, and documented, response to LFN exposure: proliferation of the extra-cellular matrix^{18,19,20}. These degenerative processes of the collagen bundles are lesions that, again, evidence that LFN-induced pathology is of the nature of collagenous disease. Cases of lupus and scleroderma, as well as immunoserological markers, have already been identified by different teams in individuals exposed to occupational and environmental LFN^{5,6,21,22}, and reproduced in animal models²³.

LFN-induced pathology is not a popular topic, nonetheless, the data presented herein demonstrate that this genotoxic agent targets the respiratory epithelia in rats. Older data gathered on LFN-exposed humans corroborate the notion the respiratory tract provides a frontline interface with LFN stress²⁴⁻²⁶. Clearly, more studies are

required until a valid hypothesis can be advanced regarding the pathophysiological mechanisms of LFN upon the respiratory epithelia of Wistar rats.

ACKNOWLEDGMENTS

The authors thank the Luso-American Foundation for Development (FLAD) for continuous support, Profs. Nuno R. Grande (ICBAS), Carlos Azevedo (ICBAS) and Carlos Sá (CEMUP) for the electron microscopy facilities, Pedro Castelo Branco for image treatment, and the extinct Occupational Medicine Research Center of the Portuguese Air Force (CIMO) for all facilities involving animal experiments. M. Alves-Pereira thanks IMAR for hosting project POCTI/MGS/41089/2001 and FCT for its funding

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