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# Chronic exposure to volcanogenic air pollution as cause of lung injury

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ABSTRACT

Few studies were made regarding the pulmonary effects of exposure to volcanogenic air pollution, representing an unrecognized health risk for humans inhabiting non-eruptive volcanically active areas (10% of world human population). We tested the hypothesis whether chronic exposure to air pollution of volcanogenic origin causes lung injury, using wild mice (*Mus musculus*) as model. Lung injury was determined using histological morphometric parameters, inflammatory status (InfS) and the amount of black silver deposits (BSD). Mice exposed to volcanogenic air pollution have decreased percentage of alveolar space, alveolar perimeter and lung structural functionality (LSF) ratio and, increased alveolar septal thickness, amount of BSD and InfS. For the first time it is evidenced that non-eruptive active volcanism has a high potential to cause lung injury. This study also highlights the usefulness of *M. musculus* as bioindicator species, and of the developed biomarker of effect LSF ratio, for future animal and/or human biomonitoring programs.



## 1. Introduction

Volcanism is an important natural source of pollutants impacting on health and environmental quality (Hansell and Oppenheimer, 2004), since in volcanically active environments levels of certain compounds are much greater than expected [e.g. yearly, volcanoes are responsible for the release of almost 150 million tons of carbon dioxide into the atmosphere (Amaral and Rodrigues, 2011)]. Moreover, about 10% of humans live in the vicinity of an active volcano, enlightening the importance of studying volcanogenic pollution. The island of S. Miguel (Azores – Portugal) is volcanically active, with major production and emission of gases and other substances that daily affect the quality of the environment and the health of human populations (Amaral et al., 2006; Amaral and Rodrigues, 2007; Rodrigues et al., 2012). According to Viveiros et al. (2010), the Furnas volcano (S. Miguel Island – Azores) emits about 1000 tons a day of carbon dioxide ($CO_2$) from soil degassing. Other main volcanic gaseous emissions include hydrogen sulfide ($H_2S$) and Radon ($^{222}Rn$). A study by Silva et al. (2007) for $^{222}Rn$ measurements was performed in 175 points (made at 50–100 cm depth) at Furnas Caldera and revealed values up to 110 KBq $m^{-3}$, being the average value of 6702 Bq $m^{-3}$. In 1999, Baxter et al. measured the indoor Rn levels in 23 houses in Furnas village and, found that combined results ranged from 140 Bq $m^{-3}$ in summer to 329 Bq $m^{-3}$ in winter (the highest reached value was 8330 Bq $m^{-3}$ in a main ground floor bedroom), which are many times more than the reference level (100 Bq $m^{-3}$) for indoor air recommended by WHO (WHO, 2009). Radon and its decay products have been shown to induce mutations (Jostes, 1996; Sethi et al., 2012), chromosome damage (Brooks et al., 1995), cell transformation or killing (Sethi et al., 2012) and immune system alterations (Nagarkatti et al., 1996).

Carbon dioxide is considered an inert asphyxiant gas and some recent studies have suggested that it may have significant bioregulatory properties, by altering neutrophil activity and behavior (Norozian et al., 2011). Although central to the inflammatory response, neutrophils are implicated in the pathogenesis of some respiratory diseases, such as emphysema (Janoff, 1985) and in cases of severe asthma (Nakagome and Nagata, 2011). Neutrophil activation is known to increase the levels of several cytokines which then leads to the migration of other leukocytes, including lymphocytes (Megiovanni et al., 2006). Therefore, it is possible that a constant activation of neutrophils leads to mobilization of lymphocytes and stimulates the appearance of macrophages (Akahoshi et al., 2003). Subsequent amplification of the pulmonary inflammatory responses is mediated by the production of a variety of leukocyte chemotactic and activating cytokines and immune

*Abbreviations:* PAlvS, Percentage of Alveolar Space; AlvP, Alveolar Perimeter; AlvST, Alveolar Septal Thickness; LSF ratio, Lung Structural Functionality ratio; InfS, Inflammatory Status; BSD, Black Silver Deposits.

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mediators (Sibille and Reynolds, 1990). In fact, several authors have also stated an increase in alveolar macrophages commonly associated with exposure to PM (Happo et al., 2010; Xu et al., 2012) and, that this increase, often related with neutrophil recruitment, negatively affects the respiratory mechanics, causing pulmonary impairment (Riva et al., 2011; Saunders et al., 2010).

Furnas Village is considered a volcanically active environment, where hazardous gases and heavy metals are present in a daily basis (Amaral et al., 2007, 2008; Viveiros et al., 2010). Studies by Amaral and Rodrigues (2007) and Amaral et al. (2006) evidenced that Furnas inhabitants have high incidence of chronic bronchitis and of some cancer types (e.g. lip and oral cavity), which may be related to chronic exposure to volcanic emissions. Moreover, a recent study by Rodrigues et al. (2012) showed an association between chronic exposure to volcanically active environments and the occurrence of DNA damage in human buccal epithelial cells of Furnas inhabitants, revealing that non-eruptive active volcanism is a risk factor of carcinogenesis. Therefore, in order to assess the effects of exposure to air pollutants of volcanogenic origin on the extent of lung injury, this study was carried out using a bioindicator species (*Mus musculus* L.) as model. The extent of lung injury was assessed by studying the following histological morphometric parameters: percentage of alveolar space (PAlvS), alveolar perimeter (AlvP) and septal thickness (AlvST). By using these parameters a lung structural functionality ratio was also determined. The inflammatory status (InfS) and amount of black silver deposits (BSD) within the lung tissue were also considered in the evaluation of lung injury.

## 2. Material and methods

Three separate sets of *M. musculus* were caught alive in the following sites of S. Miguel island (Azores, Portugal):

- Furnas village (volcanogenic site), our designated site for exposure to volcanogenic air pollutants, is a rural location with 1500 inhabitants which was built upon actively degassing ground inside a volcanic crater, where fumarolic fields and hydrothermal vents are current manifestations of volcanism and, are the cause for ongoing natural exposure to high levels of heavy metals and gases (Baxter et al., 1999; Viveiros et al., 2010).
- Ponta Delgada city (anthropogenic site), our designated site for exposure to anthropogenic air pollutants, is an urban location with nearly 60 000 inhabitants and is the most populated city in the Azores archipelago, in which road traffic is the main responsible of the emissions of air pollutants.
- Rabo de Peixe village (control site), with 5000 inhabitants, is a rural location like Furnas village, but without any type of volcanic manifestations since the seventieth century (Carvalho, 1999). This village has no apparent source of pollutants.

*Mus musculus* is considered a good bioindicator since it shares the same habitat with humans (including the houses) and due to the fact that mice population is usually large (Timm, 1994). Also, according to Forsyth (2001) and Tersago et al. (2004), studies based on field caught specimens represent a more accurate measurement of pollution exposure and accumulation patterns, since laboratory and field conditions may vary due to differences in diet, behavior or mixtures of pollutants in the environment. Furthermore, since the major source to volcanogenic pollutants is soil degassing, mice are also exposed during the day when hidden inside the holes, though they are preferentially nocturnal.

For each site, 10 adult mice were caught using live-catch mousetraps, in which the mouse would be housed no more time than the necessary to be euthanized. Only mice with a minimum weigh of 10 g were used and the average (±SE) mice weight per site was similar (13.6 ± 0.93, volcanogenic; 13.1 ± 1.19, anthropogenic; 13.5 ± 0.72; control; $F = 0.074$; df = 2; $P = 0.929$; 1-way ANOVA). Both males and females were captured, being the sex-ratio (≈50%) similar between sites. Euthanization was made with chloroform, followed by a necropsy with the extraction of the lungs. Right and left lungs were then fixed in 4% buffered formaldehyde for histological processing. This study was carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

The set of histological slides for the morphometric measurements and for the evaluation of the inflammatory status were then prepared and, consisted of 10 sections of 5-μm thickness, per slide, of each lung for every mouse. The slides were then stained with hematoxylin and eosin (H&E) in the standard manner (Martoja and Martoja-Pierson, 1970).

Another set of histological slides with one section each was prepared for AutoMetalloGraphy (AMG), which is a histochemical technique based on the principles of photography and that is used to demonstrate metals in tissue sections, following the methodology by Soto et al. (1998), by which metallic ions are revealed as BSD.

### 2.1. Lung injury: histological morphometric evaluation

AlvST was measured at 100 locations for each test subject (50 measures in each lung); measurements were made between two adjacent alveoli at a 400× magnification.

AlvP was measured at 20 (10 per lung) randomly chosen microscopic field locations for each test subject. A similar procedure was used to assess the PAlvS within each microscopic field. These measures were made with a 100× magnification, within a microscopic field area of 307 200 $\mu m^2$, and using an image analyzer (Image Pro-Plus 5.0 by MediaCybernetics®) coupled to a microscope (Leica® DM1000, Cambridge, UK). For AlvP and PAlvS within each observed microscopic field, the segments with less than 75 μm were not accounted due to the setup characteristics of the image analyzer software. Lung structural functionality (LSF) ratio was calculated by multiplying the PAlvS by the AlvP and then dividing the product by the AlvST. For each subject and morphometric parameter, averages of the left and right lungs were considered.

### 2.2. Lung injury: inflammatory status and amount of black silver deposits

The status of interstitial inflammation, based on the extent of hypercellularity and leukocyte infiltration, was assessed by double-blinded rank evaluations (based on the methodology of MacCarrick et al., 2010). Slides were read and scored from 1 to 4 according to the percentage of lung tissue with interstitial inflammation, as follow: 1–0 to 5%; 2–6 to 20%; 3–21 to 50% and 4–>50%. The amount of BSD were assessed and scored using the same double-blinded rank evaluation.

The InfS and extent of BSD were observed in 18, 20 and 20 microscopic fields of the left and right lungs (2 per mouse) of subjects from volcanogenic-polluted ($n = 9$), anthropogenic-polluted ($n = 10$) and control ($n = 10$) sites, respectively.

### 2.3. Statistical analysis

Differences between the lung AlvST, AlvP, PAlvS and LSF ratios were compared by two-way ANOVA using site (volcanogenic, anthropogenic and control) and lung side (left and right lungs) as main factors. When ANOVA showed significant differences ($P < 0.05$) between data sets, paired comparisons of each mean were made using Tukey HSD tests. Data regarding the extent of InfS and amount of BSD were compared using the non-parametric test Kruskal–Wallis. Where statistical differences existed for distribution function between data sets ($P < 0.05$), Mann–Whitney *U* tests were used to separate the differing groups. To test the association between the studied variables, Pearson's correlations were done for AlvST, PAlvS and AlvP, while Spearman's rank correlations were performed between these variables and, InfS and amount of BSD. All statistical analyses were made using SPSS 15.0 (SPSS Inc., Chicago, USA).

## 3. Results

### 3.1. Histological morphometric evaluation

PAlvS was significantly affected by the site ($F = 4.264$; df = 2; $P = 0.019$), while neither the lung side ($F = 0.013$; df = 1; $P = 0.908$) nor the interaction between these factors ($F = 0.469$; df = 2; $P = 0.628$) affected significantly this parameter.

PAlvS measured in individuals exposed to anthropogenic pollution (42.47 ± 2.41%) was significantly lower ($P = 0.022$) than in those from the control site (50.62 ± 1.21%). Although no significant differences were observed between PAlvS in individuals exposed to volcanogenic pollutants and the control site ($P = 0.082$), the mean value of the former was lower (43.96 ± 2.47%), suggesting that PAlvS is decreased in mice exposed to air polluted sites (Fig. 1A).

Similarly, AlvP was significantly affected by the site ($F = 3.901$; df = 2; $P = 0.026$), while neither the lung side ($F = 0.008$; df = 1; $P = 0.930$) nor the interaction between these factors ($F = 0.139$; df = 2; $P = 0.871$) affected significantly this parameter.

AlvP measured in the individuals exposed to volcanogenic pollutants (38 678 ± 1864 μm) was significantly lower