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Anti-oxidants reduce the acute adverse effects of residual oil fly ash on the frog palate mucociliary epithelium ☆

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Abstract

There is evidence indicating that oxidants play a pivotal role in determining air pollution-dependent lung injury. In the present study we explored the role of oxidants present in ambient particles in causing damage to the mucociliary epithelium. We explored the protective effects of pretreatment with three substances (*n*-propyl gallate, DL-α-tocopherol acetate, and EDTA) on the frog palate exposed to residual oil fly ash (ROFA). The parameters analyzed were mucociliary transport (MCT) and ciliary beating frequency (CBF) after 0, 10, 20, 30, 60, and 120 min of exposure. MCT was decreased significantly by ROFA ($P<0.001$), with a significant interaction effect ($P = 0.02$) between the duration of exposure and treatment with antioxidants. The inhibitory effects on MCT of the substances tested were significantly different ($P = 0.002$); vitamin E was similar to control (Ringer) and different from all other groups. CBF showed no significant effect of duration of exposure ($P = 0.465$), but a significant interaction between duration of exposure and treatments was observed ($P = 0.011$). Significant differences were detected among treatments ($P<0.001$), with ROFA and *n*-propyl gallate at concentrations of 50 μM presenting a short-lived increase in CBF, which was not observed in the remaining groups. The results showed that both MCT and CBF were affected within a short period (100 min) of exposure to ROFA and that the presence of antioxidant substances, such as vitamin E (4 mg/mL) and *n*-propyl gallate (300 μM), protected against the mucociliary impairment induced by ROFA on the frog palate.

Keywords: Antioxidants; Ciliary beating frequency; Mucociliary transport; Residual oil fly ash

1. Introduction

Epidemiological studies have demonstrated that the inhalation of increased levels of particulate air pollution is associated with a decline in lung function, increased respiratory symptoms, and increased morbidity and mortality in susceptible populations (Dockery and Pope, 1994; Pope et al., 1995; Schwartz, 1994).

Ambient air contains a range of pollutants, the exact combination varying from one microenvironment to the next. Despite the variability in the composition of polluted atmospheres, there is clear evidence that ambient levels of air pollution trigger pulmonary and systemic inflammation (Koenig et al., 2003; Saldiva et al., 2002; Schwartz, 2001; Souza et al., 1998).

The mucociliary apparatus represents the first line of defense of the lungs against inhaled noxious agents by removing particles and chemical species from the airways by means of the continuous transport of the airway mucus to the oropharynx using the mechanical input provided by the coordinated beating of the cilia

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(Macchione et al., 1998, 1999; Rivero et al., 2001). In this context, the mucociliary apparatus represents a point of important interaction between the defenses of the organism and inhaled toxicants, and the result of such contact influences the development of the adverse respiratory effects promoted by air pollution.

Several lines of evidence indicate that oxidants play a pivotal role in determining air pollution-dependent lung injury (Kelly, 2003). Ambient particles are able to induce oxidative stress in biological systems, either directly by the presence of oxidant substances adsorbed onto their surface or by the existence of soluble metals, including transition metals, that are capable of redox cycling (Li et al., 1996). Exposure of phagocytic cells to ambient particles collected from different urban settings causes oxidative stress that correlates with the iron content of the particles (Costa and Dreher, 1997).

We have previously shown that the frog palate preparation is an efficient experimental tool to assess the deleterious effects of oxidant stress on the ciliated epithelium (Macchione et al., 1998). In this experimental system, ambient particles were shown to induce impairment of mucociliary transport (MCT) as well as the depletion of antioxidant defenses (Macchione et al., 1999). In the present study we decided to further explore the role of oxidants present in particles in damaging the mucociliary epithelium by investigating the protective effects of pretreatment with three substances, two of them of known antioxidant capability (*n*-propyl gallate and DL-α-tocopherol acetate) and one a metal chelator (EDTA), on the frog palate exposed to residual oil fly ash (ROFA).

2. Materials and methods

2.1. Characterization of ROFA

ROFA was collected from the electrostatic precipitator of a steel plant. Trace analysis (As, Br, Ca, Co, Cr, Cs, Fe, Hf, Rb, Sb, Sc, Se, Th, U, Zn, La, Ce, Nd, Sm) was carried out by neutron activation (Table 1). The ROFA solution was obtained by ultrasonication (50 min) in Ringer solution mixed with distilled water at a 1:1 proportion to adapt its osmolarity for use in the frog palate.

2.2. Frog palate preparation

Mature frogs (*Rana catesbiana*) weighing approximately 100 g were obtained from the Mogi das Cruzes City vivarium. In our laboratory the animals received a balanced diet and water ad libitum according to routine veterinary procedures until the time for sacrifice.

Using hypothermia as anesthesia, the frogs were rapidly decapitated, their jaws were disarticulated, and the palates were removed by cutting through from the junction of the posterior pharynx and esophagus to the skin of the back. The excised palates were placed on a piece of gauze saturated with Ringer. The palates were placed on a dish loosely covered with plastic wrap and allowed to stand in a refrigerator at 4 °C for 2 days (Rubin, 1999). On the third day mucus samples were collected from the posterior edge of the palates with a needle and immediately immersed in mineral oil to prevent dehydration. All experiments were performed on the third day. Under these experimental conditions the ciliary activity is maintained (Macchione et al., 1998).

Table 1
Concentrations of trace elements in ROFA determined by neutron activation analysis (μg/g, *Ca, Fe%)

Element	ROFA (SD)
As	61.0±1.0
Br	1482±19.0
Ca*	5.42±0.16
Co	9.96±0.25
Cr	107.7±1.4
Cs	9.96±0.25
Fe*	44.6±0.1
Hf	0.62±0.07
Rb	719.7±1.0
Sb	2.27±1.0
Sc	1.91±0.01
Se	154.91±0.8
Th	1.50±0.06
U	2.28±0.14
Zn	491.9±3.1
La	10.3±0.1
Ce	16.3±0.3
Nd	9.3±1.6
Sm	2.30±0.07

2.3. In vitro mucociliary transport

MCT was determined by measuring the rate of displacement of autologous mucus samples placed on the epithelial surface of the frog palate using a stereoscopic microscopic equipped with a reticulated eyepiece. MCT was calculated by dividing the distance traveled (6 mm) by the elapsed time (*s*). At least five measurements were made for each dose and duration of the study. The mucus samples were rinsed with petroleum ether to remove the oil prior to their placement on the surface of the palate. The experiments were carried out at room temperature (20 °C). During the measurements of the MCT the frog palate was kept inside an acrylic chamber, with a microenvironment of 100% humidity provided by ultrasonic nebulization of standard Ringer solution (Macchione et al., 1998).

2.4. Ciliary beating frequency

Ciliary beating frequency (CBF) was measured by a modification of the videoscopic technique described by Braga (1988). Briefly, the technique consists of focusing on a group of cilia through a light microscope (10 × objective, 10 × eyepiece) connected to a video camera (Sony, Model 3CCD Iris), with the resulting image being sent to a monitor (Sony Trinitron). A stroboscopic light (Machine Vision Strobe, Model 5000, USA) is placed in front of the ciliary epithelium and emits flashes (0–30 Hz). The incident light illuminating the ciliated epithelium is reflected from the cilia packed together and from the thin layer of mucus covering the cilia. This reflection is cyclic, because its direction changes according to the movements of the underlying cilia. By manual control it is possible to define the frequency of ciliary activity when it is the same as the flash frequency and the observer cannot distinguish the ciliary beat.

2.5. Experimental design

Sixty frog palates were submitted to nebulization with Ringer solution for 50 min. After this period, measurements of baseline MCT and CBF were performed. After the baseline determinations, the palates were assigned to six groups and immersed in the following test solutions: negative control, (Ringer group, $n = 10$), 50 μM *n*-propyl gallate (propyl 50 group, $n = 10$), 300 μM *n*-propyl gallate (propyl 300 group, $n = 10$), EDTA (EDTA group, 75 g/1000 g body wt, $n = 10$), vitamin E (vit E group, 4 mg/mL, $n = 10$), and ROFA (ROFA group, 1 mg/mL, $n = 09$). For the propyl 50, propyl 300, EDTA, and vit E groups, additional measurements were performed 10 and 20 min after the palates were transferred to the Ringer solution containing the antioxidant substances. The negative control and ROFA groups were also measured at the same experimental times, but were kept in Ringer solution. This new set of measurements was designed to determine the influence of antioxidants on the parameters of interest, whereas the negative control and ROFA groups gave information about the stability of our measurements when palates submitted to nebulization were transferred to an aqueous medium. After these new baseline determinations, all groups, except the negative control, were transferred to Ringer solution containing ROFA (1 mg/mL), and CBF and MCT were then determined after 10, 30, and 60 min of exposition to ROFA.

Thus, we made measurements at 10 and 20 min (Ringer or pretreatment with antioxidants) and then at 30 (10 min of exposure to ROFA), 60 (30 min of exposure to ROFA), and 120 min (100 min of exposure to ROFA). Schematic representation of the experimental design is shown in Fig. 1.

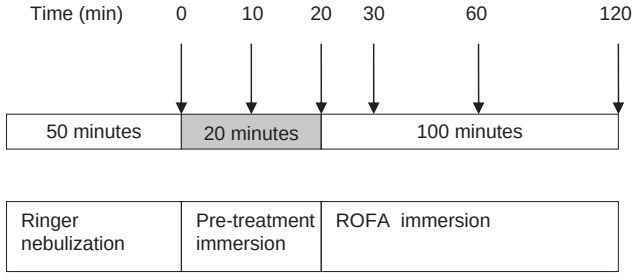

Fig. 1. Schematic representation of the experimental protocol. Pretreatment was performed with Ringer, *n*-propyl gallate (50 and 300 μM), EDTA, and vitamin E. Arrows represent the time of MCT and CBF measurements.

2.6. Statistical analysis

The statistical analysis of the results of MCT and CBF was performed using general linear models for repeated measurements containing categorical indicators of treatment as well as a term for duration and treatment interaction. Post hoc tests were also employed (least significant difference) for multiple comparisons. The level of significance was set at 5%. Statistical analyses were performed with the aid of the SPSS v10.0 computer package.

3. Results

Table 2 presents the descriptive statistics of the measurements of MCT determined for all experimental durations and treatments. The same data are depicted graphically in Fig. 2. Exposure to ROFA induced a decrease of MCT, which increased with duration of exposure ($P < 0.001$). The effects of duration of exposure exhibited a significant interaction with the treatments employed ($P = 0.02$). The effects on MCT of the substances tested were significantly different ($P = 0.002$), with the effects of Ringer and vitamin E differing from those of the other groups.

Table 3 and Fig. 3 present the results obtained in terms of CBF measurements. Exposure to ROFA induced a decrease of CBF, which started after 10 min of exposure (Time 30) and lasted until the end (Time 120) of our measurements ($P = 0.011$). This decrease was affected by the different treatments employed ($P < 0.001$), with ROFA and propyl 50 differing from the remaining groups.

4. Discussion

The contribution of air pollution to the severity of respiratory diseases has been repeatedly reported in both epidemiological (Abbey et al., 1995; Pope et al., 2002)

Table 2
Descriptive statistics of mucociliary transport (mm/s) for all experimental groups

	Time (min)	Mean	SEM	95% Confidence interval	
				Lower bound	Upper bound
ROFA	0	0.53	0.41	0.45	0.62
pretreatment	10	0.47	0.30	0.41	0.53
	20	0.46	0.03	0.40	0.52
ROFA	30	0.42	0.035	0.35	0.48
	60	0.35	0.04	0.28	0.42
	120	0.24	0.04	0.17	0.32
Propyl 50	0	0.49	0.04	0.41	0.57
pretreatment	10	0.47	0.03	0.41	0.53
	20	0.45	0.03	0.39	0.51
ROFA	30	0.44	0.03	0.37	0.50
	60	0.41	0.03	0.34	0.47
	120	0.32	0.04	0.25	0.39
Propyl 300	0	0.47	0.04	0.39	0.55
pretreatment	10	0.43	0.03	0.37	0.48
	20	0.43	0.29	0.37	0.49
ROFA	30	0.47	0.03	0.41	0.54
	60	0.39	0.03	0.32	0.45
	120	0.31	0.04	0.24	0.38
EDTA	0	0.49	0.04	0.42	0.57
pretreatment	10	0.42	0.03	0.37	0.48
	20	0.41	0.03	0.35	0.47
ROFA	30	0.44	0.03	0.37	0.50
	60	0.39	0.03	0.32	0.45
	120	0.33	0.03	0.26	0.40
Vit E	0	0.48	0.04	0.41	0.56
pretreatment	10	0.53	0.03	0.47	0.59
	20	0.49	0.03	0.43	0.55
ROFA	30	0.58	0.03	0.51	0.64
	60	0.42	0.03	0.36	0.49
	120	0.41	0.04	0.33	0.48
Ringer	0	0.57	0.04	0.49	0.64
pretreatment	10	0.56	0.03	0.50	0.61
	20	0.55	0.03	0.49	0.61
ROFA	30	0.53	0.03	0.46	0.59
	60	0.42	0.03	0.46	0.59
	120	0.51	0.04	0.44	0.59

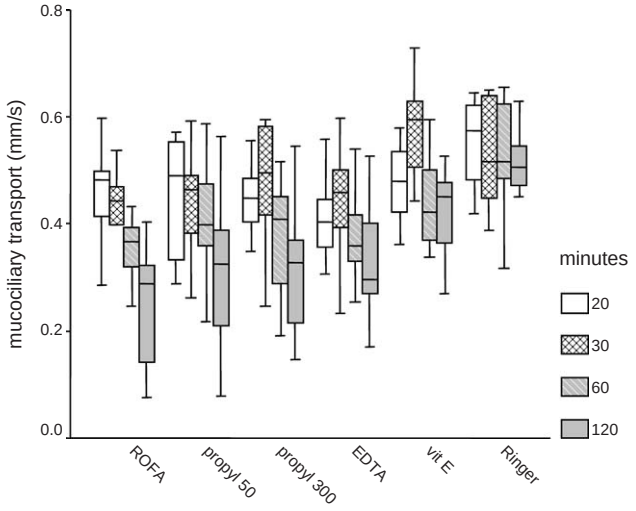

Fig. 2. Box plot representing the values of MCT (in mm/s) determined for all experimental treatments and times 20, 30, 60, and 120. Abbreviations used: MCT, mucociliary transport; ROFA, residual oil fly ash; propyl 50, *n*-propyl gallate 50 μM; propyl 300, *n*-propyl gallate 300 μM; EDTA, ethylenediaminetetraacetic acid; vit E, DL-α-tocopherol acetate.

and experimental studies (Cendon et al., 1997; Lemos et al., 1994; Reymão et al., 1997; Saldiva et al., 1992). Particulate air pollution has been reported to contribute to pulmonary disease in many studies because it contains a higher proportion of various toxic metals and acid sulfur species (Pope et al., 1991). There is now a substantial body of evidence showing that oxidative stress mediated by metals is a major contributor to the toxic effects of the particulate air pollution on the respiratory apparatus (Kelly, 2003). Experimental studies have demonstrated that exposure to ROFA is highly toxic to the lungs (Dreher et al., 1997) and that ROFA extracts are able to induce oxidative stress and inflammation in pulmonary tissue (Costa and Dreher, 1997; Kadiiska et al., 1997).

In previous studies, we demonstrated that the frog palate is affected by levels of oxidants in the range expected to occur in the inflammatory milieu (Macchione et al., 1998). In the presence of ambient particles, we observed evidence of impaired mucociliary function, as well as depletion of antioxidant defenses, suggesting that oxidative stress plays a role in mucociliary dysfunction. We reasoned that if the oxidant damage was the main factor responsible for the mucociliary dysfunction observed in our previous studies, this dysfunction should be abolished or diminished when antioxidant substances are added to the model. Our data showed that both MCT (Table 2) and CBF (Table 3) were impaired after a short exposure to ROFA. Similar effects have been observed in our laboratory in the same model exposed to H_2O_2 and PM_{10} (Macchione et al., 1998, 1999). The capacity of ROFA to cause lung injury is remarkable in animal models (Pritchard et al., 1996; Norwood et al., 2001; Ghio et al., 2002a, b). Since ROFA is rich in metals, with few organic components, it has been particularly useful as a surrogate for testing the hypothesis that metals mediate the biological effects of air pollution particles (Ghio et al., 2002a, b).

Our results showed that the presence of antioxidant substances, such as vitamin E, and also of the higher

Table 3
Descriptive statistics of ciliary beating frequency (Hz) for all experimental groups

	Time (min)	Mean	SEM	95% Confidence interval	
				Lower bound	Upper bound
ROFA	0	14.78	0.58	13.63	15.94
pretreatment	10	16.36	0.44	15.48	17.23
	20	16.10	0.53	15.03	17.16
ROFA	30	14.16	0.40	13.37	14.96
	60	14.76	0.39	13.98	15.55
	120	14.59	0.43	13.72	15.46
Propyl 50	0	15.57	0.55	14.47	16.67
pretreatment	10	15.93	0.42	15.09	16.76
	20	16.26	0.50	15.25	17.27
ROFA	30	16.98	0.38	16.23	17.74
	60	15.98	0.37	15.23	16.73
	120	15.59	0.41	14.77	16.42
Propyl 300	0	14.58	0.55	13.48	15.67
pretreatment	10	13.58	0.42	12.74	14.41
	20	13.92	0.50	12.91	14.93
ROFA	30	14.12	0.38	13.36	14.87
	60	15.24	0.37	14.50	15.99
	120	14.81	0.41	13.98	15.63
EDTA	0	13.84	0.52	12.88	14.88
pretreatment	10	14.42	0.40	13.62	15.21
	20	14.23	0.48	13.27	15.20
ROFA	30	14.65	0.36	13.93	15.37
	60	14.10	0.36	13.39	14.81
	120	14.92	0.39	14.13	15.70
Vit E	0	13.48	0.55	12.75	14.94
pretreatment	10	14.56	0.42	13.72	15.39
	20	14.56	0.50	13.55	15.57
ROFA	30	15.41	0.38	14.67	16.16
	60	14.86	0.37	14.12	15.61
	120	14.65	0.41	13.83	15.48
Ringer	0	14.48	0.55	13.39	15.58
pretreatment	10	14.31	0.42	13.48	15.15
	20	14.05	0.50	13.31	15.06
ROFA	30	13.74	0.38	12.98	14.49
	60	13.78	0.37	13.03	14.53
	120	13.95	0.41	13.13	14.78

dosage of *n*-propyl gallate protects against the mucociliary impairment induced by ROFA. These findings support the concept that the mechanisms involved in mucociliary impairment are at least in part mediated by oxidative stress.

Although the results obtained in this acute investigation are in agreement with many experimental studies showing that oxidative stress is an important mechanism of injury on the airway epithelium

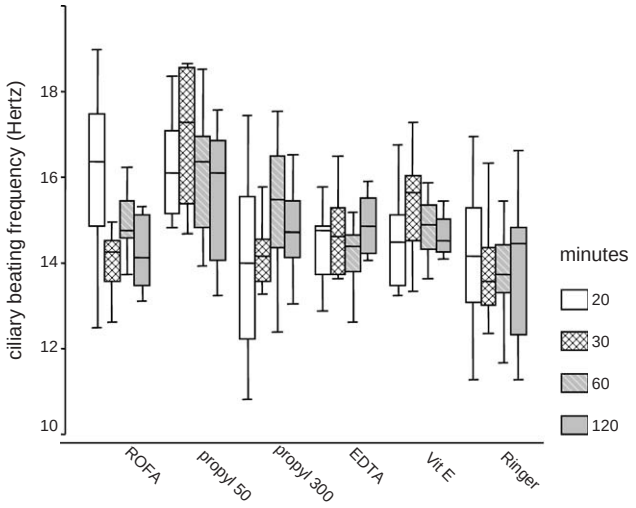

Fig. 3. Box plot representing the values of CBF (in Hz) determined for all experimental treatments and times 20, 30, 60, and 120. Abbreviations used: CBF, ciliary beating frequency; ROFA, residual oil fly ash; propyl 50, *n*-propyl gallate 50 μM; propyl 300, *n*-propyl gallate 300 μM; EDTA, ethylenediaminetetraacetic acid; vit E, DL-α-tocopherol acetate.

(Costa and Dreher, 1997; Kadiiska et al., 1997; Li et al., 1996; Macchione et al., 1999), the extrapolation of these experimental data to the field of ambient conditions is difficult. First, ROFA is probably a much more potent toxic agent than regular ambient aerosol (Ghio et al., 2002a, b). Second, the dose of inhaled particles after dilution in the microenvironment surrounding the respiratory epithelium in vivo does not correspond to that employed in our investigation. The first interface that a pollutant encounters when it enters the airway is the lung-lining fluid. This fluid consists of secretions from the underlying lung and resident immune cells, as well as plasma-derived substances. Studies with bronchoalveolar lavage have shown that lung-lining fluid contains a range of low-molecular-weight antioxidants similar to that of blood plasma, including reduced glutathione, ascorbic acid, uric acid, and α-tocopherol (vitamin E) (Kelly, 2003). Despite the limitations, the demonstration that antioxidants inhibit the acute, deleterious effects of ROFA on the mucociliary system reinforces the concept that oxidants play a role in determining respiratory effects induced by particles produced by oil combustion.

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