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Research Article

Short-term Exposure to Concentrate Ambient Particle Induces Myocardial Fibrosis

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Abstract

Epidemiological and experimental studies have consistently shown that both short- and long-term exposure to air pollution is associated with a variety of cardiovascular diseases. Air pollution is composed of a mixture of noxious substance including particles and gases, and its adverse effects are more commonly attributed to fine particulate matter (PM2.5). As the association of air pollution and cardiorespiratory diseases is a global concern, some important issue still remains unknown. Thus, we investigated the effects of different time exposure (8, 15 and 22 days) to concentrated ambient particles (CAP) derived from vehicle emissions on both physiological and morphofunctional parameters of the heart in normal juvenile rats. Our data have displayed that air pollution exposure is capable to negatively impact cardiac structure and function by increasing heart rate variability (HRV), left ventricular wall thickness and myocardial fibrosis in the left ventricle. In conclusion, our results have shown that particulate air pollution induces changes in the autonomic control of the heart and that ambient traffic-derived particles are capable in a short period of inducing changes in the microstructure of the myocardium.

Keywords: *Particulate Air Pollution; Heart; Stereology; Myocardial Fibrosis; HRV*

Abbreviations

PM2.5: Fine Particulate Matter; CAP: Concentrated Ambient Particles; HRV: Heart Rate Variability; PM: Particulate Matter; AVMA: National and Institutional Guidelines for Animal Welfare; CAPPesq-HCFMUSP: Ethics Committee for Analysis of Research Projects from Hospital das Clínicas of University of Sao Paulo Medical School; AF: Filtered Air; HEPA: High-Efficiency Particulate Arrestance, HAPC: Harvard Ambient Particle Concentrator; BNC: Bayonet Neill-Concelman; NIBP Non-Invasive Blood Pressure; BP: Blood Pressure; HR: Heart Rate; HRV: Heart Rate Variability; SDNN: Standard Deviation of NN Intervals; RMSSD: Root Mean Square of Successive Differences; LF: Low Frequency; HF: High Frequency; EDTA: Ethylene Diamine Tetraacetic Acid; BAL: Bronchoalveolar Lavage; PBS: Sodium Phosphate Buffer; SUR: Systematic Uniform Random; HE: Hematoxylin-Eosin; VT: Total Volume; Vv: Volume Fraction; LV: Left Ventricle; VWMV: Volume-Weighted Mean Volume; HIF-1α: Hypoxia-Inducible Factor 1 Alpha; TGF-β1: Transforming Growth Factor Beta 1; BSA: Bovine Serum

Albumin; DAB: 3`3-Diaminobenzidine; SPSS: Statistical Package for Social Sciences; ANOVA: Analysis of Variance; CO: Carbon Monoxide; PAH: Polycyclic Aromatic Hydrocarbons

Introduction

Air pollution in large urban centers has been increasing at levels due to large industrial activity, increase in circulating vehicles and degradation of natural resources. Among the several pollutants present in the air, particulate matter (PM) is one of the main responsible for the harmful effects on human health [1]. Its toxicity is directly related to its diameter, small size (particles < 10; 2.5; 0.1 μm) and its great ability to move through the upper respiratory tract, reaching the lungs and other organs [2].

Epidemiological and experimental evidence has shown that air pollution in urban cities is a risk factor for many diseases. This issue has been explored by several authors and studies demonstrated that the exposure to PM can increase the risk of neurodegenerative diseases [3-5], promotes cardiac remodeling [6], increases heart rate [7,8], increases pulmonary fibrosis [9], increased expression of pro-inflammatory molecules and systemic inflammation [10,11].

Cardiovascular disease represents an important public health problem [12,13]. The fine particulate matter ($PM_{2.5}$) is considered one of the determinant factors on the onset and aggravation of this disease in urban communities [14-18]. Studies have shown that PM exposure was significantly associated with cardiovascular mortality [19,20], as well as increased hospitalizations due to cardiovascular diseases [21].

The mechanisms through which particles can affect the cardiovascular system have been revised elsewhere [15] and the main pathophysiological responses include systemic oxidative stress and inflammation, thrombosis and coagulation, hypertension, atherosclerosis and altered vascular reactivity [22].

Differently from our study, previously published studies that explored the association between air pollution and cardiovascular diseases used invasive techniques (intratracheal or intranasal) of pollutant administration which does not mimic real environmental conditions to which humans are exposed. In addition, particles properties could be modified by dilution preparation. As pointed by Kodavanti., *et al.* [23], most of these studies evaluated the effects of particles on cardiophysiology [24-27] and less attention was given to histopathology or biochemical alterations of the myocardium. Therefore, the aim of this study was to evaluate both physiological and morphofunctional effects of different time of exposure (8, 15 and 21 days) to real urban fine particulate matter on the heart of juvenile healthy rats.

Materials and Methods

Animals and experimental groups

All experiments were conducted in accordance with national and institutional guidelines for animal welfare (AVMA) [28] and approved by the Ethics Committee for Analysis of Research Projects from Hospital das Clínicas of University of Sao Paulo Medical School (CAPPesq-HCFMUSP number 0839/09). Animals were treated humanely with special concern to the alleviation of distress and discomfort.

We studied male Wistar rats obtained from the Animal Facility of the University of Sao Paulo Medical School with an initial age of 3 months. These rats were divided into 6 groups (n = 5 - 6 per group) and were daily exposed to filtered air (AF) or concentrated ambient particles (CAP) for 8, 15 or 22 consecutive days. Before the exposure beginning, animals were submitted to an adaptation period of one week. Groups are described as follow:

- AF-8: Animals exposed to filtered air for 8 consecutive days;
- AF-15: Animals exposed to filtered air for 15 consecutive days;
- AF-22: Animals exposed to filtered air for 22 consecutive days;

- CAP-8: Animals exposed to concentrated ambient particles for 8 consecutive days;
- CAP-15: Animals exposed to concentrated ambient particles for 15 consecutive days;
- CAP-22: Animals exposed to concentrated ambient particles for 22 consecutive days.

During the exposure period, animals were accommodated in exposure chambers within their own cages where they were free to move and breath. Water and food were given *ad libitum*. Outside the exposure periods animals were maintained in a light and dark cycle of 12hs:12hs in special racks with High-Efficiency Particulate Arrestance (HEPA) filters, thus receiving only filtered air.

Exposure protocol and characterization of the particulate matter

The exposure to CAP was conducted using a Harvard Ambient Particle Concentrator (HAPC) which is located in the garden of the University of Sao Paulo Medical School, Brazil, close to a busy traffic crossroad. This apparatus has a series of virtual impactors that select $PM_{2.5}$ and vacuum pumps that direct $PM_{2.5}$ to the exposure chambers [29]. CAP groups were daily exposed to a dose of 600 $\mu g/m^3$ of $PM_{2.5}$. AF groups were daily exposed to filtered air.

Everyday sample filters were collected and the elemental analysis was performed to characterize the composition of the CAP. This analysis was performed using an X-ray fluorescence spectrometry (EDX 700 HS, Shimadzu Corporation, Japan) as described by Veras and colleagues [30].

Electrocardiographic parameters

Electrocardiographic data were collected one day before the beginning of the exposure period and then one day before the euthanasia (at days 7, 14 and 21 of exposure). Animals were intraperitoneally anesthetized with xylazine (9 mg/Kg) and ketamine (42 mg/Kg). A rodent-specific cuff and pulse transducer were applied to the base of the tail and connected via BNC (Bayonet Neill-Concelman) to a digital data acquisition NIBP (Non-Invasive Blood Pressure) system [ML125/R PowerLab system, AD Instruments, Mountain View, CA]. The following parameters were analyzed: blood pressure (BP), heart rate (HR) and heart rate variability (HRV) through time-domain methods (standard deviation of NN intervals [SDNN] and root mean square of successive differences [RMSSD]) and frequency-domain methods (low frequency [LF], high frequency [HF], and LF/HF ratio).

Hemogram

After the end of the exposure periods, (at days 8, 15 and 22 of exposure) rats were weighed and euthanized by intraperitoneal injection of pentobarbital (200 mg/kg). Blood was collected from the abdominal aorta artery by punction. Blood sampling was collected in EDTA (Ethylene diamine tetraacetic acid) K3 tubes for the analyses of erythrogram (erythrocytes, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume and reticulocyte count) and leukogram (leukocytes, neutrophils, eosinophils, basophils, lymphocytes, and monocytes). The hematological analysis was performed by using Pentra 120 from ABX Diagnostics (Montpellier, France). All these procedures were performed in the Hematology Service of the Central Laboratory of Hospital das Clínicas of University of Sao Paulo Medical School.

Bronchoalveolar lavage (BAL)

After blood collection, trachea and lungs were removed, weighed and placed in a dish soaked in sodium phosphate buffer (PBS, pH 7.4). A catheter was inserted into the trachea and 3 lavages were made with 2.5 ml of PBS. A final volume of 7.5 ml was centrifuged at 1810 rpm, 5°C for 10 minutes. The supernatant was frozen at -70°C. The pellet was resuspended in 1000 µl of PBS. 100µl of the resuspended pellet was removed and placed in a tube with 400 µl of PBS. 10 µl of BAL were packed in the Neubauer hemocytometer for total cell count by 400x magnification. For the differential count, 100 µl of BAL were added in cytospin (Cytospin 3 - Shandon) and cytocentrifuged at 450 rpm for 6 minutes. The material deposited on the blade was stained with Diff-Quick (Baxter Dade, Dudingen, Switzerland) for differential

counting of macrophages, neutrophils, lymphocytes, and eosinophils under an optical microscope with immersion objective and 1000x magnification.

Morphological analysis

During euthanasia, heart, liver, spleen, kidney, brain, and lungs were collected and weighted. Atriums were removed from the heart and the ventricles were weighted. The total volume of ventricles (VT_{VENT}) was obtained by dividing the weight of ventricles (g) by the specific density of the tissue (1.06g/cm^3) [31].

The sampling procedure for histological analysis of the ventricles was systematic uniform random (SUR) [32]. Six to eight slices of 2 mm were produced per ventricle. Then, the slices were fixed by immersion in 4% paraformaldehyde solution for 24 hours and maintained in 70% ethanol until processing. The slices were processed for paraffin embedding and paraffin blocks were sectioned at 5 µm. One section per slice was collected onto glass slides and stained with Hematoxylin-Eosin (HE).

The slides were scanned using the software Pannoramic™ Viewer v. 1.15 (3DHISTECH Ltd.; Budapest, Hungary). Low magnification photomicrographs (2x) were used to estimate the volumes of ventricle compartments. High magnification photomicrographs (40x) were used to assess the histological compartments of the left ventricle. The photomicrographs were analyzed using the software ImageJ (http://rsb.info.nih.gov/ij).

Stereological analysis

To assess the volumes of compartments, a point test grid was superimposed on the photomicrographs and points falling on each specific compartment were counted. The volume fraction (Vv) was quantified in 40 fields randomly obtained from all sections. For low magnification, right and left ventricle, and septum were counted. For high magnification, cardiomyocytes, connective tissue, and vessels were counted [33]. The Vv of each compartment was obtained by applying the following formula:

$$Vv = \sum pt_{COMP} / \sum pt_{VENT}$$

Where $\sum pt_{COMP}$ is the sum of points falling on the specific compartment and $\sum pt_{VENT}$ is the sum of points falling in all compartments.

For low magnification, the total volume of each compartment was obtained by multiplying the Vv by the total volume of the ventricles (VT_{VENT}). For high magnification, the total volume of each histological compartment of the left ventricle was obtained by multiplying the Vv by the total volume of the left ventricle (VT_{LV}).

Individual volumes of cardiomyocytes were obtained indirectly. Volume-weighted mean volume (VWMV) of cardiomyocytes was estimated using stereologic point-sampled intercept method as previously described by Gundersen and Jensen [34]. A mean of 30 randomly fields were examined for each heart. A line with point test grid was superimposed on the fields and intercepts were measured along the test lines of the grid from cell boundary to cell boundary. Only cardiomyocytes hit by points were sampled. The lengths of cell intercepts (l) were then applied to the following formula to obtaining the VWMV of cardiomyocytes:

$$VWMV = \pi * l_0^3 / 3$$

When a cardiomyocyte was hit for more than once by a point, the same number of cell intercepts measurements were done and the mean value of them included in the formula.

Morphometric analysis

Left ventricle wall thickness (µm) was estimated directly by measuring its thickness in three different positions in all slices. A total of 18 - 24 measures were obtained per heart.

The diameter of cardiomyocytes (μm) was obtained directly. Cardiomyocytes were randomly selected and the largest and the smallest diameter were measured directly. Nearly 100 cardiomyocytes were measured per animal. The mean of both measurements was considered as the cardiomyocyte's diameter.

Collagen analysis

Collagen content in the myocardium was evaluated with Picro-Sirius Red [35]. For every section, measurements were performed on 15 random fields (20x magnification). Collagen analysis was performed with Image-Pro® Plus 4.5 for Windows® software (Media Cybernetics, Silver Spring, MD, USA) on a computer connected to a digital camera coupled to a Leica DMR light microscope (Leica Microsystems Wetzlar GmbH, Germany). A spectrum range of red color was previously determined and used to select automatically the collagen stained with Picro-Sirius Red. Given SUR sampling of sections, area fractions of positively stained tissue correspond to volume fractions of collagen (%). Subsequently, volume fractions were converted into absolute volumes of collagen (cm^3) by multiplying by the total volume of ventricles (VT_{VENT}).

Immunohistochemistry analysis

Immunohistochemistry of ventricles was conducted for Caspase 3, Hypoxia-inducible factor 1 alpha (HIF-1α) and Transforming growth factor beta 1 (TGF-β1). Paraffin blocks were sectioned at a 5 μm thickness on a microtome. Sections were deparaffinized, hydrated and endogenous peroxidase was blocked. Antigen retrieval was performed with heated citrate buffer and unspecific bindings were blocked with bovine serum albumin (BSA). Primary antibody incubations were performed separately with 1:500 of caspase-3 antibody (Santa Cruz Biotechnology [sc-7148]), 1:150 of HIF-1α antibody (Santa Cruz Biotechnology [sc-53546]) and 1:400 of TGFβ1 antibody (Santa Cruz Biotechnology [sc-146]). The Vectastin ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody and 3`3-Diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) was used as the chromogen. Sections were counterstained with Harris hematoxylin. For negative controls, incubations were performed with BSA.

Immunohistochemistry analysis was performed with Image-Pro® Plus 4.5 for Windows® software (Media Cybernetics, Silver Spring, MD, USA) on a computer connected to a digital camera coupled to a Leica DMR light microscope (Leica Microsystems Wetzlar GmbH, Germany). Given SUR sampling of sections, area fractions of positively stained tissue correspond to volume fractions (%). Subsequently, volumes fractions were converted into absolute volumes by multiplying by the total volume of ventricles (VT_{VENT}).

Statistical analysis

Statistical analysis was performed using the software SPSS (Statistical Package for Social Sciences) version 17.0. Means, standard errors and standard deviations were calculated for each group and parameter. One-way analysis of variance (ANOVA) was used to compare the outcomes among the six groups and multivariate analysis was used to observe the influence of factors (exposure and time) on the outcomes. Null hypotheses were rejected at a probability level of $p < 0,05$.

Results

Elemental composition of the PM2.5

The evaluation of the elemental composition of $PM_{2.5}$ showed that 97% of the mass consisted of elemental carbon and the remaining 3% of the mass fraction is distributed between different elements as shown in figure 1.

Heart rate, heart rate variability, and blood pressure

HRV for time-domain variables, SDNN and RMSSD, were significantly higher in animals exposed to CAP ($p = 0.01$ and $p = 0.02$) compared to the control group. Moreover, a significant effect was found in SDNN in animals exposed to CAP for 22 days compared to the non-exposed rats.

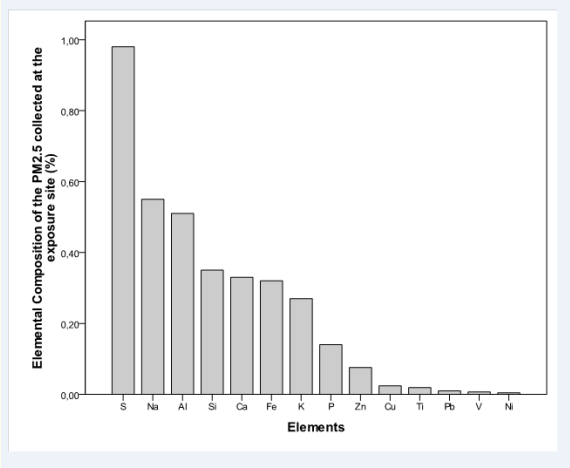

Figure 1:*Elemental composition of PM2, 5 from the environment. S, sulfur; Na, sodium; Al, aluminum; Si, silicon; Ca, calcium; Fe, iron; K, potassium; P, phosphorus; Zn, zinc; Cu, copper; Ti, titanium; Pb, lead; V, vanadium; Ni, nickel.*

Frequency domains analysis of HRV had shown significantly increase over time in LF and HF in animals exposed to CAP (p = 0.001 and p = 0.04, respectively). However, the association LF/HF did not show any difference. Regarding the number of beats per minute (HR), the exposure to CAP was able to significantly decrease HR (p = 0.003). The arterial pressure (AP) was not affected by exposure to CAP (these data are shown in table 1).

Parameter	Filtered Air (n = 23)	CAP (n = 27)	P
HR (bpm)	285.27 ± 31.673	259.85 ± 30.687	0,003
Time-domain			
SDNN (ms)	26.43 ± 19.237	36.48 ± 22.755	0,01
RMSSD (ms)	31.91 ± 22.603	43.67 ± 26.558	0,02
Frequency-domain			
LF (ms^2)	104.13 ± 119.055	227.67 ± 259.716	0,001
HF (ms^2)	77.35 ± 86.500	135.30 ± 133.890	0,04

Table 1: *Electrocardiographic parameters of animals exposed to filtered air or CAP. Data are expressed as mean ± SD. CAP: Concentrated Ambient Particles, HR: Heart Rate, SDNN: Standard Deviation of NN Intervals, RMSSD: Root Mean Square of Successive Differences, LF: Low Frequency, HF: High Frequency.*

Body and organs weight

The body weight evaluation of control and exposed groups are presented in figure 2. While in the control group were observed a significant increase in the weight gain throughout time (p = 0.02), the animals exposed to CAP did not show any difference. Moreover, spleen, lungs, brain, and kidney weighted immediately after the euthanasia did not show any difference. However, liver weight was affected (p = 0.02) by the interaction of exposure and time (p = 0.004). After 8 days of exposure liver from CAP-8 group presented more weight gain compared to the control group, but as exposure continues the weight of the liver seemed to be reduced (Figure 3).

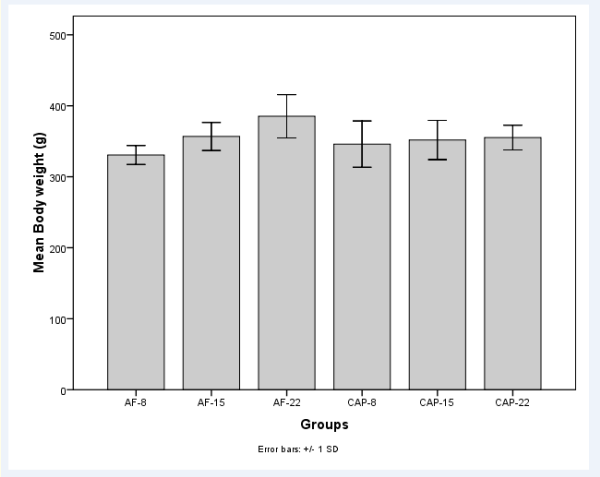

Figure 2: *Mean body weight (g) of animals exposed to filtered air (AF) or concentrated ambient particles (CAP).*

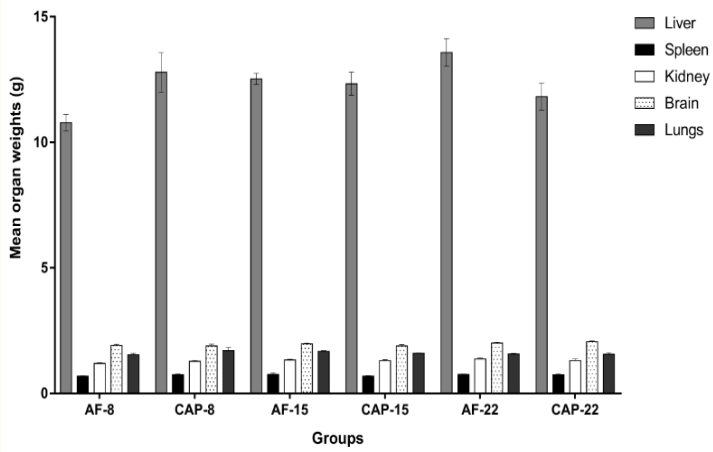

Figure 3: *Mean lungs, liver, kidney and spleen weight (g) of animals exposed to filtered air (AF) or concentrated ambient particles (CAP) during different period of exposure (8, 15 and 22 days).*

Hemogram and BAL analysis

No differences were found neither for hemogram nor bronchoalveolar lavage.

Morphological parameters

Table 2 shows that exposure to $PM_{2.5}$ is associated with an increase in the volume density of connective tissue in the ventricles ($p = 0.029$) as well as a decrease in volume fraction of cardiomyocytes ($p = 0.006$) at 15 days in animals exposed to CAP when compared to the control group. Regarding the total volume, there was a significant increase in the total volume of connective tissue of the heart at 15 days

in animals exposed to air pollution when compared to control group (p = 0.002). Figure 4 shows a histological section of the left ventricle myocardium of rats exposed to CAP or filtered air at different time of exposure.

	Volume fraction (Vv)			Total volume (VT)		
Group	**Cardiomyocytes (%)**	**Connective (%)**	**Vessels (%)**	**Cardiomyocytes (cm^3)**	**Connective (cm^3)**	**Vessels (cm^3)**
AF-8	0.94 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.39 ± 0.13	0.02 ± 0.02	0.01 ± 0.01
AF-15	0.96 ± 0.01	0.00 ± 0.00	0.03 ± 0.01	0.46 ± 0.17	0.00 ± 0.00	0.02 ± 0.01
AF-22	0.93 ± 0.03	0.03 ± 0.03	0.04 ± 0.00	0.52 ± 0.05	0.02 ± 0.02	0.02 ± 0.00
CAP-8	0.90 ± 0.02	0.06 ± 0.02	0.03 ± 0.00	0.38 ± 0.13	0.03 ± 0.01	0.01 ± 0.00
CAP-15	0.81 ± 0.05*	0.17 ± 0.05*	0.02 ± 0.01	0.57 ± 0.06	0.11 ± 0.03*	0.02 ± 0.01
CAP-22	0.84 ± 0.05	0.13 ± 0.05	0.03 ± 0.02	0.51 ± 0.04	0.08 ± 0.04	0.02 ± 0.01

Table 2: *Mean volume fraction (Vv) and total volume (VT) of heart tissue compartments. Data are expressed as mean (%) ± SD or mean (cm^3) ± SD for cardiomyocytes, connective tissue and vessels. Difference is significant at p < 0.05*. Vv: Volume Fraction, VT: Total Volume, AF: Filtered Air, CAP: Concentrated Ambient Particles.*

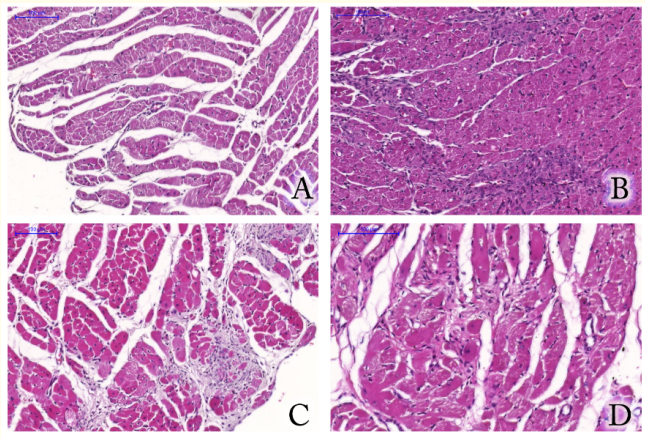

Figure 4: *Photomicrographs (20x) of histological section of the left ventricle myocardium of rats exposed to concentrated ambient particles or filtered air at different times of exposure stained by Hematoxylin-Eosin method. (A) Control animal (AF-15); (B) Animal exposed to CAP for 8 days; (C) Animal exposed to CAP for 15 days; (D) Animal exposed to CAP for 22 days.*

The evaluation of the average thickness of the free wall of the left ventricle showed borderline significant differences between the groups (p = 0.05). We observed that animals exposed to CAP (CAP-8 and CAP-15) presented a slight increase in free wall thickness (mean ± SD; AF-8 = 1976 ± 452; CAP-8 = 2595 ± 303; AF-15 = 1737 ± 289; CAP-15 = 2540 ± 466) when compared to air filtered groups, however, differences at 22 days of exposure were not observed. No other difference was found for the parameters analyzed.

Collagen analysis

In the sections stained with Picro-Sirius Red were found significantly increase (p = 0.010) in the percentage area occupied by collagen in the myocardium of exposed animals when compared by the control group (data not shown). Also, the exposure to CAP significantly

increased the collagen total volume (cm^3) (p = 0.009) in animals exposed to CAP in all time of exposure evaluated. This result is presented in figure 5. No other differences were found in this analysis. Figure 6 shows histological sections of the left ventricle myocardium stained with Picro-Sirius Red in control and exposed animals.

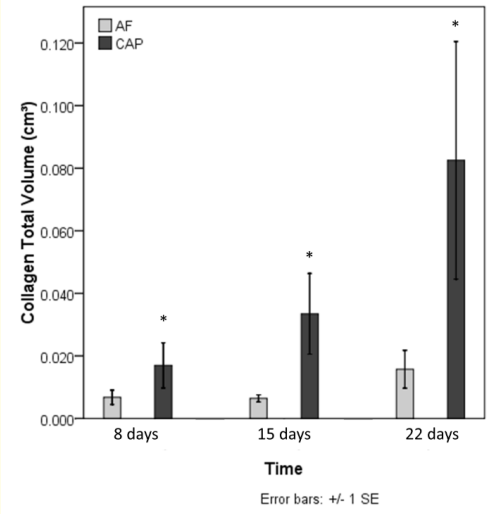

Figure 5: *Graphic representation of total volume of collagen (cm^3) in the heart of animals exposed to filtered air (AF) or concentrated ambient particles (CAP) during different period of exposure (8, 15 and 22 days). *p = 0,009 (CAP x AF).*

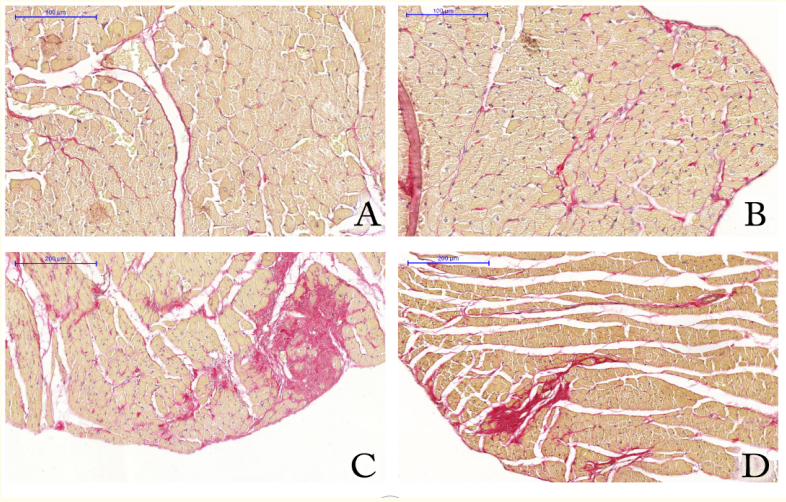

Figure 6: *Photomicrographs (20x) of histological section of the left ventricle myocardium of rats exposed to concentrated ambient particles or filtered air at different times of exposure stained by Picro-Sirius Red method. (A) Control animal (AF-15); (B) Animal exposed to CAP for 8 days; (C) Animal exposed to CAP for 15 days; (D) Animal exposed to CAP for 22 days.*

Immunohistochemistry analysis

No differences were found to HIF-1α neither TGF-β1 among the groups. Only the number of cells marked positively with Caspase-3 per area was found to be significantly different ($p = 0.039$) when analyzed by exposure. Animals exposed to CAP had a smaller number of cells/area when compared to the control group (data not shown).

Discussion

In this study, we evaluated the effects of short-term exposure to CAP on both physiological and morphofunctional parameters of Wistar rat's hearts. Our data are in accordance with previous experimental studies and epidemiological findings indicating that urban particulate air pollution is an environmental risk for cardiovascular health impairment. We have shown that a short period of the daily accumulated exposure dose (600 $\mu g/m^3$ - daily annual mean concentration = 28 $\mu g/m^3$) [36] in which individuals are exposed in São Paulo city is capable to negatively impact cardiac structure and function by increasing heart rate variability, left ventricular wall thickness and myocardial fibrosis in the left ventricle.

We conducted the experiments using an HAPC [37] which allowed us to expose animals only to particulate pollution with an aerodynamic diameter less than 2.5 μm (size cut 0.1 - 2.5 μm) in order to exclude the interaction effect of other pollutants or larger particles. Air pollution in urban centers is composed by a mixture of gaseous substances (nitrogen oxides, sulfur oxides, carbon monoxide) and particles of different size (coarse [PM_{10}], fine [PM < 2.5] and ultrafine [PM < 0.1] particles), mainly emitted by vehicles [38]. Inhaled $PM_{2.5}$ enters the blood circulation via diffusion and it can reach deeper into the lungs, kidneys, and heart [39]. Thus, $PM_{2.5}$ pose the greatest risk to health [38] and it is more commonly associated with cardiovascular effects [40].

The association of air pollution and cardiorespiratory diseases is a global concern [41] and their effects on the cardiovascular system has been recognized since many years ago [42]. Studies from across the world have consistently shown that both short- and long-term exposures to fine PM are associated with cardiovascular diseases [15,43]. Some effects are observed shortly after exposure to high levels of particulate air pollution, such as, increase blood pressure, arterial vasoconstriction, change the vascular tone and blood coagulability [44]. Other outcomes, such as heart rate variability, arterial pressure, vascular tone, and blood coagulability have been also reported [15,45-47].

In addition, previous experimental studies from our research group have shown that mice chronically exposed to urban air pollution presented mild but significant vascular structural alterations such as coronary arteriolar fibrosis and elastosis [36], thickening of the arterial wall in the coronary and pulmonary circulation [48], heart edema [49] and vasoconstriction of pulmonary arterioles after 14 days of exposure [50].

Most of the published studies indicate that air pollution is associated with decreased HRV both in animals and in humans, including intratracheal exposure to particulate matter [51-55]; however, our data showed that HRV was increased in animals exposed to CAP. Although there is a consensus that decreased SDNN and RMSSD are associated with higher cardiovascular mortality risks [56,57], there are cases in which patients that present increased HRV also present elevated risks of heart failure [58,59].

Similarly to the results presented here, but using animals exposed to biodiesel, Brito., *et al.* found that a daily average of 22.9 $\mu m/m^3$ showed an increase in HRV in Balb/c mice [60]. Still, in accordance with our results, Elder., *et al.* reported an elevation of HRV and increased HR in rats exposed to highway aerosol in comparison to clean air-exposed rats [61]. The highway aerosol also affected short-term alterations in autonomic control of HR. These results support the idea of traffic-related ultrafine particles is a potential to affect the autonomic nervous system.

In humans, Tarkiainen., *et al.* examined the association between acute carbon monoxide (CO) exposure and cardiac autonomic function by measuring HRV in patients with stable coronary artery disease. The CO exposure was associated with elevated RMSSD, also sug-

gesting that exposure derived from traffic may increase vagal control during CO exposure [62]. Shields., *et al.* observed a positive association between HRV parameters (SDNN, HF, and LF) and a relatively low concentration of $PM_{2.5}$ (mean $PM_{2.5}$ mass concentration = 14 μg/m^3) in a middle-aged population in Mexico City, while the LF/HF ratio was negatively associated with that pollutant [46]. Although there are some discrepant results, these combinations of findings provide some support for the idea that changes in HRV (both increases and decreases) can be associated with both ambient and traffic-related particulate matter. Moreover, the synergy between $PM_{2.5}$ and ambient gaseous co-pollutants may potentialize the effects on health and this is an important issue because of the mixture of fine PM and gaseous pollutants are different from the mixture of the background air pollution by which individuals are exposed [46,63].

It is also important to point out that although many advances have already been made in the use of HRV as a prognostic factor of cardiovascular risk in humans, the use of this parameter in laboratory animals, especially rodents, is still new and a standardized protocol still needed to be determined [64,65]. Further, experimental protocols present great differences between methods of exposure, duration of the exposure, origin, and composition of particles and outcomes evaluated [24,26,27,66].

In our study, we tried to mimic as close as possible real-world exposure to $PM_{2.5}$. Thus, a whole-body exposure was performed to avoid the effects of stress (e.g. restraint in nose-only exposure) and modification in particle composition due to solution preparation for tracheal instillation. The key finding of the present study was the fact that exposure to CAP was capable to induce myocardium fibrosis after a short period of exposure. Myocardial fibrosis is a diffuse and disproportionate accumulation of collagen in the myocardial interstitium. That condition contributes to LV dysfunction, arrhythmia, impaired myocardial oxygen availability and consequent heart failure [67].

Several studies have reported an increase in myocardial fibrosis after exposure to air pollution [6,23,68-72]. Kodavanti., *et al.* in 2003, in their study with Wistar-Kyoto rats showed that protracted, repeated inhalation exposure to oil combustion-derived particulate matter (high concentrations) led to degenerative and fibrotic lesions in the myocardium [23]. Further, in 2008, Kodavanti and his colleagues indicated that PM-associated zinc appears to be an important contributor to myocardial degeneration and fibrosis after 16 weeks of intratracheal instillation. They suggest that these findings might be explained by the fact that PM exposure stimulates systemic endothelin release and microvascular thrombosis resulting in myocardial injury [68]. In addition, exposure to diluted motorcycle exhaust led to increased wall thickness and fibrosis in rats [71].

Still corroborating our study, Akinaga., *et al.* found that chronic exposure (4 months) to ambient air pollution ($PM_{2.5}$) of Swiss mice from birth to adulthood induced fibrosis in LV and affected all large-sized arteries [36]. Similarly, as an indicative of fibrosis, de Oliveira-Fonoff and colleagues demonstrated increased collagen deposition in both right and left ventricles of Wistar rats exposed to CAP by 4 weeks (28 days) [6] and Tanwar., *et al.* showed that also in utero exposure to $PM_{2.5}$ could lead to an increase of collagen deposition at left ventricle, accompanied by increased mRNA expression of the gene *Col-1* (collagen production), what suggests an epigenetic effect of air pollution [72]. Although fibrosis has been characterized as a long-term effect that occurs after inflammation processes to repair tissue injuries [73], in our study we observed increased collagen deposition even at short-term (8, 15 and 22 days) exposure to CAP, suggesting that air pollution could also induce negative acute effects on the cardiac tissue structure.

Despite the literature shows several studies corroborating our study, the use of different protocols and animal models might have some interference in the results. Moreover, we did not evaluate effects on cardiomyocytes and cardiac fibroblast at the molecular level, so further studies are needed to better explain our findings.

Elemental composition of $PM_{2.5}$ sampling collected confirmed that the main source is vehicle emissions [74-76]. Within elements, different transition metals are present (Al, Fe, Zn, Cu, Ti, and Vn), which are known to affect the cardiorespiratory and immune system [77,78].

We have also found Pb in our samples and it leads to toxicity on the heart structure and function is already well know. Even at low doses, Pb affects cardiovascular function both in humans and animals. Chronic exposures to low levels of Pb induces changes in the elec-

tric and mechanical activity of the heart and degenerative alterations of the myocardium [79-81]. Although we did not quantify the PAHs (polycyclic aromatic hydrocarbons) and oxidants gases, it is important to mention that these pollutants also contribute to the hazardous effects observed in the heart.

Conclusion

In conclusion, this study has a significant public health relevance in demonstrating that short- term exposure to exclusively fine particulate matter ($PM_{2.5}$) at environmentally relevant concentration is associated with histopathological changes in normal rats. Future studies on the current topic are therefore recommended to identify molecular networks involved in the short-term exposure to air pollution and its effects on the heart's failure, functionality, and morphology.

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