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# Cardiac autonomic regulation after lung exposure to carbon nanotubes

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**The ultrafine (UF) component of airborne pollution may impair cardiovascular autonomic control, a high-risk condition for cardiovascular adverse events. Since engineered nanoparticles, such as single-walled carbon nanotubes (SWCNTs) share physicochemical properties with UF, they might have similar adverse effects. Aim of the study was to evaluate arterial baroreflex function (BRF) at baseline, 24 h after the first instillation, immediately before the second one, and 2 weeks later, in adult Wystar-Kyoto conscious rats undergoing two intratracheal instillations of SWCNT (eight rats) or phosphate buffer saline (PBS) (five rats) at 2-week interval. During each session, 30-min continuous recording of arterial pressure and pulse interval was performed by a telemetered catheter implanted in the abdominal aorta of the rats. BRF was studied by the sequence technique. SWCNTs dispersed in PBS (1 mg/ml) were administered immediately after sonication (1 µg/g body weight). A significant decrease in the number of baroreflex sequences (from 498 ± 27.1 at baseline to 287 ± 40.2 at the recording performed after 4 weeks; $P < 0.05$) was observed in SWCNT-instilled rats, whereas no significant change was detected in controls. These data suggest that SWCNTs may alter the BRF, thus affecting the autonomic cardiovascular control regulation.**

**Key words:** arterial baroreflexes; cardiac autonomic control; cardiovascular toxicity; single-walled carbon nanotubes

## Introduction

There is strong evidence that episodic high levels of airborne particulate matter (PM) are associated with stroke, heart attacks, arrhythmias, and sudden death [1]; these events may be precipitated, at least in part, by alterations in the autonomic input to the heart,[2–5] as evidenced by a partial or total loss in spontaneous heart rate variability (HRV). Indeed, decreased HRV is highly predictive for increased risk of arrhythmias and sudden cardiac death,[6] especially in subjects with ischemic heart disease.[7]

It has been reported in previous studies on PM that ultrafine (UF) particles are more cytotoxic, inflammatory, and fibrogenic on an equivalent mass basis than fine-sized particles of the same composition. Accordingly, a recent large European study showed that the UF component of PM is the major factor contributing to the alterations of cardiovascular autonomic control.[8] The underlying mechanisms responsible remain unclear. However, a role can be hypothesized for activation of pulmonary neural reflex arcs, direct effects of pollutants on cerebral areas responsible for autonomic control or on cardiac ion channels, or heightened systemic inflammatory state.

Synthetic nanomaterials, developed in recent years to engineer new structures, materials, and devices, generally occur in size ranges similar to UF particles, therefore rising concern about their possible adverse effects on cardiovascular system. Indeed, some experimental data suggest that carbon nanotubes, a class of synthetic nanomaterials with peculiar electrical, mechanical, and thermal properties, may promote atherosclerosis.[9] No data are currently available on their possible effects on the cardiovascular autonomic control, and on the arterial baroreflex function (BRF). This function is based on the response of cardiac heart rate (HR) to arterial pressure (AP) changes. Even minimal AP changes are recorded by specialized aortic and carotid baroreceptors, which immediately transmit the information through afferent nerve fibers to an

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integrating center in the hypothalamic region of the brain, which in turn modulates the cardiac HR through efferent nerve fibers. This system acts as a homeostatic mechanism aimed to maintain constant AP values. BRF impairment is considered predictive for cardiac mortality, especially in patients affected by ischemic heart disease and myocardial infarction.[10]

Aim of the present study was to test the hypothesis that single-walled carbon nanotubes (SWCNTs) pulmonary exposure affects the arterial BRF.

## Methods

*Animal protocol*

The study was performed on 13 adult Wystar-Kyoto (WKY) rats of both sexes obtained from Charles River Laboratories Italia (specific pathogen free (SPF), 350 g body weight) and the experimental procedures were carried out according to the Association for Assessment and Accreditation of Laboratory Care International and approved by the animal care facility (Stazione per la Tecnologia Animale) of the University "Tor Vergata" and by the Italian Health Minister. Rats were used and housed individually in the animal care facility, allowed normal rat chow and drinking water ad libitum (diet RF18), and kept on a 12 h light–12 h dark cycle. Temperature and humidity were continuously monitored (20 ± 2 °C, relative humidity (RH) 55 ± 5%). Rats were maintained in SPF conditions by using microisolator technique (Filter top cage).

After induction of anesthesia by ketamine (Ketavet 50® 60 mg/kg, i.p.) and medetomidine (Domitor® 0.3 mg/kg, i.p.), a telemetry transmitter (TA11PA-C40, Data Sciences, St. Paul, Minnesota) was implanted for recordings of AP signals according to manufacturer specifications. The tip of the arterial catheter was inserted into the abdominal aorta previously exposed by a midline incision through a hole made by a 21-gauge needle below the bifurcation of the renal arteries just proximal to the iliac bifurcation and secured in place with tissue glue (Vetbond, 3M®, 3M Medical Division, St. Paul, USA.). The transmitter body was attached to the abdominal wall along the incision line with sutures as the incision was closed. After surgery, the rats were given antibiotics (ceftriaxone) and housed individually in cages for 5–7 days, until complete recovery.

The recording system is composed of the following three basic elements: 1) a transmitter for blood pressure monitoring (TA11PAC40); 2) a receiver (RPC-1); and 3) an adapter (R11CPA) including an ambient pressure monitor (APR-1) that produces analog output signals of pulsatile AP.

The telemetered AP signal was digitized using an analog I/O PC card (National Instrument 6024E, Austin, Texas) at a rate of 2000 Hz, displayed on the computer screen and processed by an algorithm based on feature extraction to detect and measure the characteristics of AP cycles developed in our laboratory based on a Lab view platform software. Systolic arterial pressure (SAP) and diastolic arterial pressure (DAP) were calculated. Pulse interval (PI) was measured from the pressure pulses and used to calculate HR.

*Study design*

Rats were randomly divided into two groups: control rats ($n = 5$) and SWCNT-instilled rats ($n = 8$). Thirty minutes continuous AP recordings were performed at baseline (time 0), 24 h (time +24), 2 weeks (time 1), and 4 weeks (time 2) later. SWCNT or phosphate buffer saline (PBS) was given to SWCNT-instilled and control group, respectively, at time 0 and time 1, immediately after AP recording. After the induction of anesthesia, the trachea was intubated with a polyethylene cannula, and the instillation was performed.

The baroreflex function was assessed by means of the sequence analysis.[11,12] Briefly, the beat-by-beat time series of SAP and PI were analyzed by the earlier-described software in order to identify spontaneously occurring sequences of three or more consecutive beats in which SAP and PI of the fifth heart beat (i.e., lag 5, used for the high HR of rats)[13] changed in the same direction, thus resembling the classical baroreflex-mediated AP and HR changes, for example, SAP increase and PI decrease (i.e., hypertension and bradycardia) or SAP decrease and PI increase (i.e., hypotension and tachycardia). These sequences were identified as baroreflex sequences (Figure 1), similarly to the Oxford technique employing bolus injections of vasoactive drugs. Only those sequences in which $r^2$ was >0.85 were accepted and the number of baroreflex sequences was calculated.

The mean individual slope of the baroreflex sequences, obtained by averaging all slopes computed within a given experimental period, was calculated and taken as a measure of the baroreflex sensitivity (BRS) for that period. A linear regression was applied to each individual sequence.

*Chemicals*

SWCNTs (CarboLex AP-grade, 50–70%, Aldrich, Steinheim, Germany) produced by the arc-discharge

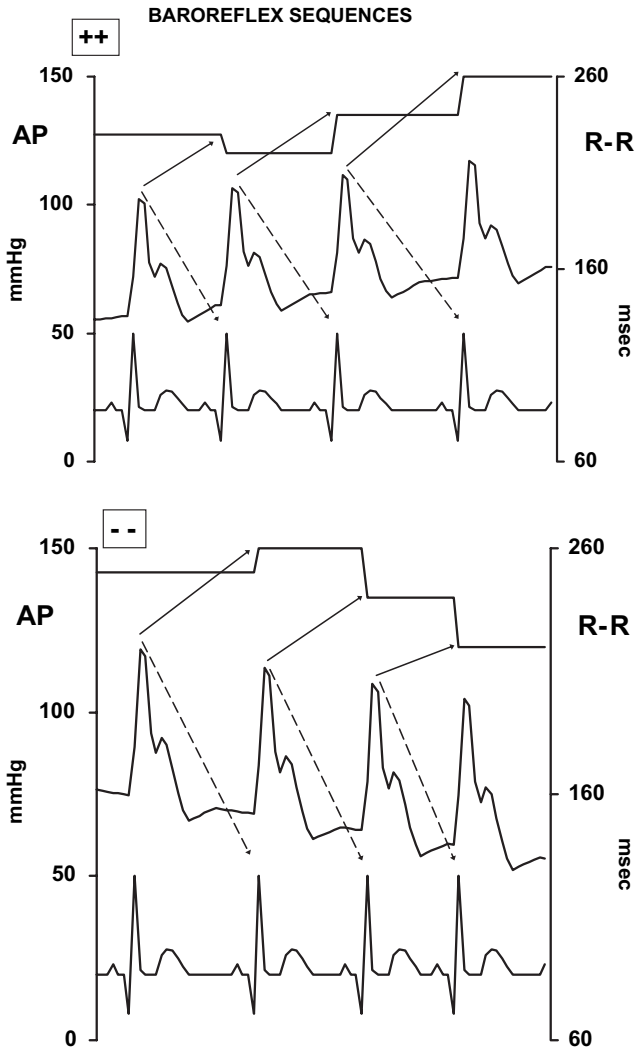


**Figure 1** Examples of the blood pressure and electrocardiogram signals of the corresponding pulse intervals. The signals refer to sequences during which SAP and PI of the following beat changed in the same direction, either increasing or decreasing (Baroreflex sequences).

process, employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor and purified by the $HNO_3$ acid treatment at room temperature, were used in this study. For the morphological and structural characterization of SWCNTs, Raman spectroscopy and scanning electron microscopy (SEM) were routinely used to characterize the as-received nanotube samples and the purified samples. The Raman spectroscopy revealed a typical spectrum associated with the radial breathing mode of carbon atoms, representing the fingerprint of SWCNT. The spectrum was unaffected by the purification procedure. Purified SWCNTs were sterilized 3 h in 160 °C before they were suspended in 0.1 M PBS (pH 7.4) at the final concentration of 1 mg/ml. The mean diameter, length, and surface area of SWCNTs were 1.2–1.6 nm, 2–5 nm, and 300 $m^2/g$, respectively. Surface area was determined by Brunauer, Emmett, and Teller analysis.

The suspensions were sonicated at 100 W by means of a Branson Sonifier B-12 (Cell Disruptor, Sonic Power Company Danbury Connecticut). We checked the stability of the suspension at different times (i.e.; 18 s, 1 min, 10 min, and 30 min, respectively) and found that 10-min sonication allowed stability up to 1 min. Therefore, during the experimental procedures, the purified SWCNT dispersion was sonicated for 10 min, and then instilled intratracheally within 1 min. The amount of SWCNT given during each session was 1 μg/g body weight. This concentration is in the lower range of the dosage given in previous experimental studies.[9,14,15]

*Statistical analysis*

AP data were stored and analyzed by a computerized on line system for biological data elaboration developed in our laboratory based on a Lab View Platform software.

Within groups, changes in the reported variables were evaluated by one-way analysis of variance (ANOVA) for repeated measures for normally distributed variables and by Kruskal–Wallis one way ANOVA on Ranks for non-normally distributed variables. The significance of differences of baseline values between the two experimental groups was evaluated by *t*-test. All data are presented as means ± SEM. A value of $P < 0.05$ was considered statistically significant.

## Results

In baseline conditions (time 0), cardiovascular parameters did not differ between control and SWCNT-instilled rats (Table 1).

AP did not show significant changes along the different experimental sets in response to repetitive exposure both in control and in SWCNT-instilled rats, whereas HR showed a trend to decrease at time 1 and at time 2 in both the groups. The change, however, was statistically significant only in SWCNT-instilled rats (Figure 2).

At baseline (time 0), the number of baroreflex sequences was lower in controls (270 ± 28.4) than in SWCNT-instilled rats (498 ± 27.1; $P < 0.05$) (Figure 3).

Interestingly, the occurrence of baroreflex sequences showed an evident and significant trend to decrease in response to SWCNT exposure

**Table 1** Cardiovascular values in baseline conditions in the two experimental groups

| | *SAP (mmHg)* | *DAP (mmHg)* | *HR (beats/min)* |
|---|---|---|---|
| Baseline controls | 134.4 ± 4.0 | 100.5 ± 2.0 | 342.1 ± 22.0 |
| Baseline SWCNT instillations | 123.6 ± 5.9 | 103.3 ± 5.6 | 332.3 ± 14.2 |

SAP, systolic arterial pressure; DAP, diastolic arterial pressure; HR, heart rate.
Mean values ± SEM.

(427 ± 45.2 after 24 h, 386.0 ± 45.0 after 2 weeks, 287 ± 40.2 after 4 weeks; $P < 0.05$ after 2 and 4 weeks) whereas the number of baroreflex sequences did not show significant changes in response to PBS instillations (273.6 ± 20.8 after 24 h, 190.0 ± 26.1 after 2 weeks, 214.0 ± 47.3 after 4 weeks; $P$ = NS for any comparison) (Figure 3). It is noteworthy that this event was not associated to a decrease in heart beat spontaneous fluctuations, as indicated by the lack of significant changes in both groups in the standard deviation of the HR time series (the main index of HRV) (Figure 4).

BRS did not show significant changes during the whole experimental period both in controls (from

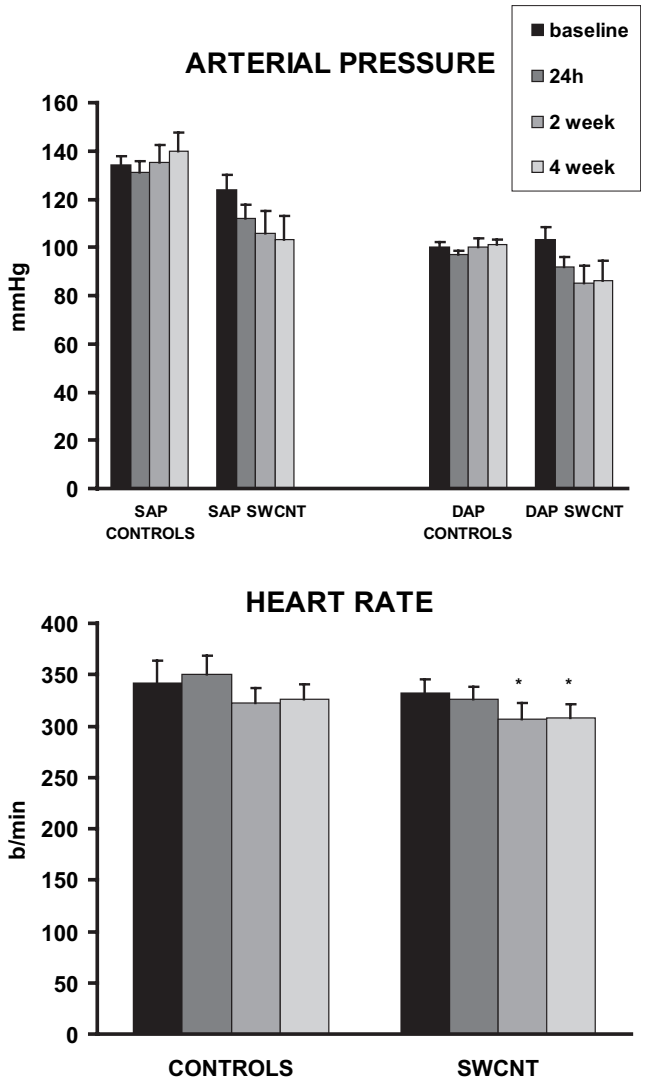


**Figure 2** Arterial pressure (upper panel) and heart rate (bottom panel) values during the PBS (Controls) and single-walled carbon nanotubes (SWCNT) instillations at different experimental time (see Methods). SAP, systolic arterial pressure; DAP, diastolic arterial pressure. Data are shown as mean ± SEM. *$P < 0.05$ vs baseline.

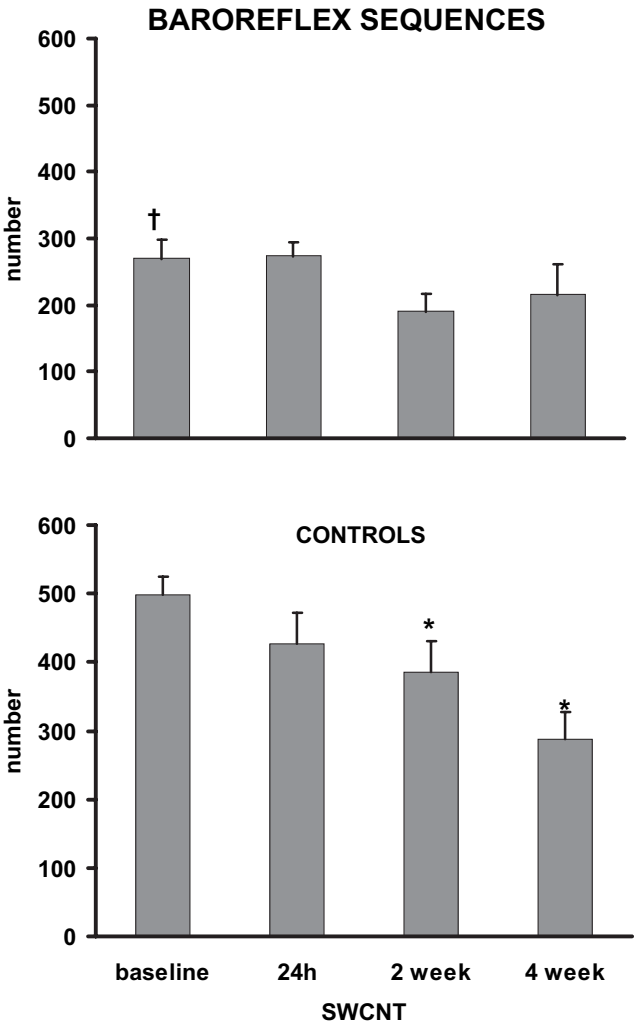


**Figure 3** Number of baroreflex sequences during the PBS (Controls, upper panel) and single-walled carbon nanotubes (SWCNT, bottom panel) instillations at different experimental time (see Methods). Data are shown as mean ± SEM. *$P < 0.05$ vs baseline. †$P < 0.05$ control vs SWCNT-instilled group baseline values.

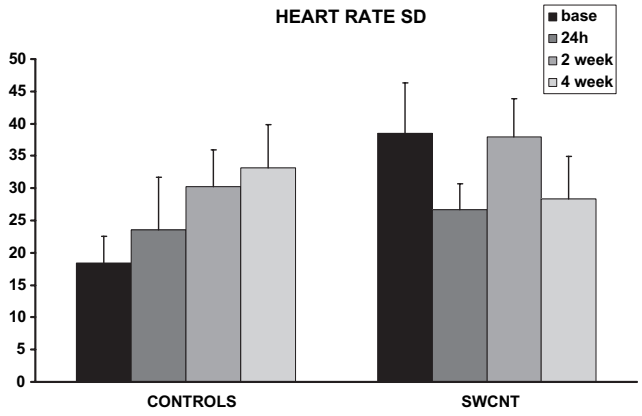


**Figure 4** Standard deviation of the heart rate time series during the PBS (Controls) and single-walled carbon nanotubes (SWCNTs) instillations at different experimental time (see Methods). Data are shown as mean ± SEM.

3.7 ± 1.3 in baseline conditions to 4.6 ± 1.5 after 24 h, 4.9 ± 1.4 after 2 weeks, 4.6 ± 0.2 ms/mmHg after 4 weeks; $P$ = NS for any comparison) and in SWCNT-instilled rats (from 5.3 ± 1.1 in baseline conditions to 4.2 ± 1.2 after 24 h, 6.3 ± 1.0 after 2 weeks, 5.7 ± 1.7 ms/mmHg after 4 weeks; $P$ = NS for any comparison) (Figure 5).

## Discussion

This is the first report on the possible influence of engineered nanoparticles on the cardiac autonomic regulation; in particular, we tested the hypothesis that repetitive exposure to SWCNT through intratracheal instillation could affect the BRF.

Our data showed a progressive significant decrease in the number of baroreflex sequences in exposed animals but not in the control group. In contrast, the gain of the baroreflex control of sinus node, as estimated by the BRS, did not show significant changes both in exposed and nonexposed animals. The data, however, were obtained from a relatively low number of animals, and cannot be considered definitive at this time; for example, there were differences at baseline in the number of baroreflex sequences between the two groups, probably due to chance and generated by the low number of animals: this fact emphasizes the need for confirmatory studies.

A peculiar characteristic of our experimental model is that it was performed in a physiological setting, that is, on unanesthetized conscious, freely moving rats. This consideration is important in the light of the great influence that external factors (e.g., drugs injections, anxiety due to restriction, anesthetics) can have on the autonomic regulation of the cardiovascular system.

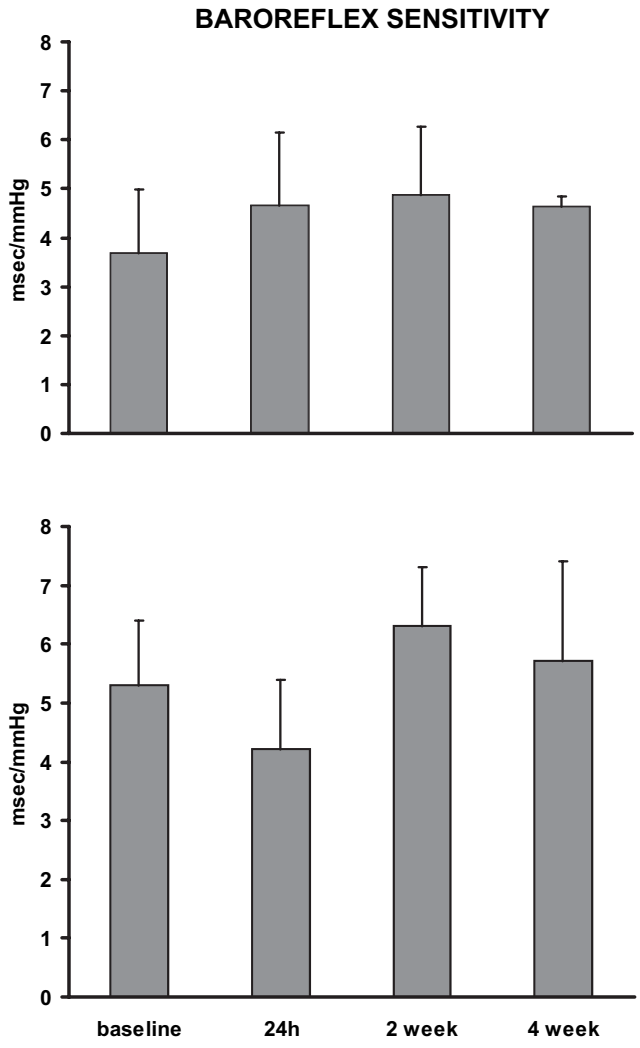


**Figure 5** Baroreflex sensitivity during the PBS (Controls, upper panel) and single-walled carbon nanotubes (SWCNT, bottom panel) instillations at different experimental time (see Methods). Data are shown as mean ± SEM.

Moreover, the relatively low amount of SWCNT used in the present study may be considered similar, on the basis of available data on respiratory dust levels during laboratory handling of SWCNT,[16] to cumulative exposures which can be reached in an occupational setting in less than 2 years.[17]

The observed pattern of modification of the BRF suggests a partial loss of sensitivity to AP and HR spontaneous fluctuations of the carotid and aortic baroreceptors, whereas the central baroreflex integration, which greatly contributes to set the gain of the arterial baroreflexes,[18] seems unaffected. Although a cumulative effect due to repetitive instillations may have contributed to this response, it is

noteworthy that the reduced occurrence of baroreflex sequences was observed before the second instillation after 2 weeks from the first exposure, and may, therefore, be interpreted as a delayed effect of the initial administration. This finding is in keeping with the biopersistence hypothesis of carbon nanotubes.[19]

Both direct and indirect mechanisms may link pulmonary exposure to SWCNT to the altered BRF. Indeed, SWCNTs are able to induce a peculiar inflammatory pulmonary reaction,[13] eliciting the release into the systemic circulation of inflammatory cytokines, which in turn may adversely affect the cardiac autonomic control.[20] However, given their very small size, SWCNT may elude pulmonary macrophage uptake, gaining direct access into the systemic circulation.[19] Therefore, a direct effect of SWCNT on specialized peripheral structures sending afferent stimuli to the brain cannot be excluded.

Some study limitations must be considered. In fact, we cannot exclude that the observed effects may be related, at least in part, to metal contaminants introduced during the process of synthesis of carbon nanotubes. Indeed, SWCNT may contain a number of toxic metals including Co, Fe, Ni, and Mo, all of which have documented toxic effects. The SWCNT used in the present study may have appreciable amounts of nikel,[21] which may not be completely removed even after the purification process. However, the expected increasing occupational and environmental exposure to carbon nanotubes will probably involve material contaminated with a variable amount of different metals, whose effects might be considered along with those of carbon nanotubes themselves in these contexts.

In our experiments, we did not evaluate the respiratory patterns of the animals during the BRF evaluation. It has been reported that pulmonary SWCNT exposure induces a persistent accumulation of carbon nanotube aggregates in the lung followed by the rapid formation of pulmonary granulomatous and fibrotic tissues at the site.[13,21] We cannot exclude that modifications in the respiratory pattern, possibly due to the alteration, induced by SWCNT pulmonary exposure might have contributed to the arterial baroreflex changes observed in our study; indeed, respiratory activity greatly influences the baroreflex control of sinus node.[22,23]

In conclusion, we have shown that SWCNT may affect the autonomic control of cardiac activity and in particular the arterial baroreflex control of sinus node. This finding, if confirmed by further studies, may have relevant implications in terms of public health, given the well known predictive role in cardiac mortality of the arterial baroreflex alterations and the expected widespread use of this material in occupational and environmental settings.

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## References

1 Mossman, BT, Borm, PJ, Castranova, V, Costa, DL, Donaldson, K, Kleeberger, SR. Mechanisms of action of inhaled fibers, particles and nanoparticles in lung and cardiovascular disease. *Part Fibre Toxicol* 2007; **4**: 4.

2 Magari, SR, Hauser, R, Schwartz, J, Williams, PL, Smith, TJ, Christiani, DC. Association of heart rate variability with occupational and environmental exposure to particulate air pollution. *Circulation* 2001; **104**: 986–991.

3 Gold, DR, Litonjua, A, Schwartz, J, Lovett, E, Larson, A, Nearing, B, *et al*. Ambient pollution and heart rate variability. *Circulation* 2000; **101**: 1267–1273.

4 Pope III, CA, Verrier, RL, Lovett, EG, Larson, AC, Raizenne, ME, Kanner, RE, *et al*. Heart rate variability associated with particulate air pollution. *Am Heart J* 1999; **138**: 890–899.

5 Liao, D, Creason, J, Shy, C, Williams, R, Watts, R, Zweidinger, R. Daily variation of particulate air pollution and poor cardiac autonomic control in the elderly. *Environ Health Perspect* 1999; **107**: 521–525.

6 Algra, A, Tijssen, JG, Roelandt, JR, Pool, J, Lubsen, J. Heart rate variability from 24 hour electrocardiography and the 2 year risk for sudden death. *Circulation* 1993; **88**: 180–185.

7 Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Heart rate variability: standards of measurement, physiological interpretation, and clinical use. *Circulation* 1996; **93**: 1043–1065.

8 Timonen, KL, Vanninen, E, de Hartog, J, Ibald-Mulli, A, Brunekreef, B, Gold, DR, *et al*. Effects of ultrafine and fine particulate and gaseous air pollution on cardiac autonomic control in subjects with coronary artery disease: the ULTRA study. *J Expo Sci Environ Epidemiol* 2006; **16**: 332–341.

9 Li, Z, Hulderman, T, Salmen, R, Chapman, R, Leonard, SS, Young, SH, *et al*. Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes. *Environ Health Perspect* 2007; **115**: 377–382.

10 La Rovere, MT, Bigger Jr, JT, Marcus, FI, Mortara, A, Schwartz, PJ. Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction. *Lancet* 1998; **351**: 478–484.

11 Legramante, JM, Raimondi, G, Massaro, M, Cassarino, S, Peruzzi, G, Iellamo, F. Investigating feedforward neural regulation of circulation from analysis of spon-

taneous arterial pressure and heart rate fluctuations. *Circulation* 1999; **99**: 1760–1766.

12 Legramante, JM, Raimondi, G, Massaro, M, Iellamo, F. Positive and negative feedback mechanisms in the neural regulation of the cardiovascular function in healthy and spinal cord injured humans. *Circulation* 2001; **103**: 1250–1255.

13 Oosting, J, Struijker-Boudier, HAJ, Janssen, JA. Validation of a continuous baroreceptor reflex sensitivity index calculated by spontaneous fluctuations of blood pressure and pulse interval in rats. *J Hypertens* 1997; **15**: 391–399.

14 Shevdova, AA, Kisin, ER, Mercer, R, Murray, AR, Johnson, VJ, Potapovich, AI, *et al*. Unusual inflammatory and fibrogenic pulmonary response to single walled carbon nanotubes in mice. *Am J Physiol Lung Cell Mol Physiol* 2005; **289**: L698–L708.

15 Shvedova, AA, Kisin, ER, Murray, AR, Gorelik, O, Arepalli, S, Castranova, V, *et al*. Vitamin E deficiency enhances pulmonary inflammatory response and oxidative stress induced by single walled carbon nanotubes in C57BL/6 mice. *Toxicol Appl Pharmacol* 2007; **221**: 339–348.

16 Maynard, AD, Baron, PA, Foley, M, Shvedova, AA, Kisin, ER, Castranova, V. Exposure to carbon nanotube material: aerosol release during the handling of unrefined single walled carbon nanotube material. *J Toxicol Environ Health* 2004; **967**: 87–107.

17 Mercer, RR, Scabilloni, J, Wang, L, Kisin, E, Murray, AR, Schwegler-Berry, D, *et al*. Alteration of deposition pattern and pulmonary response as a result of improved dispersion of aspirated single-walled carbon nanotubes in a mouse model. *Am J Physiol Lung Cell Mol Physiol* 2008; **294**: L87–L97.

18 Goldstein, B, Toweill, D, Lai, S, Sonnenthal, K, Kimberly, B. Uncoupling of the autonomic and cardiovascular systems in acute brain injury. *Am J Physiol* 1998; **275**: R1287–R1292.

19 Donaldson, K, Aitken, R, Tran, L, Stone, V, Duffin, R, Forrest, G, *et al*. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol Sci* 2006; **92**: 5–22.

20 Nolan, RP, Reid, GJ, Seidelin, PH, Lau, HK. C-reactive protein modulates vagal heart rate control in patients with coronary artery disease. *Clin Sci (Lond)* 2007; **112**: 449–456.

21 Lam, CW, James, JT, McCluskey, R, Hunter, R. Pulmonary toxicity of single wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol Sci* 2004; **77**: 126–134.

22 Eckberg, DL, Kifle, YT, Roberts, VL. Phase relationship between normal human respiration and baroreflex responsiveness. *J Physiol (Lond)* 1980; **304**: 489–502.

23 Van De Borne, P, Mezzetti, S, Montano, N, Narkiewicz, K, Degaute, JP, Somers, VK. Hyperventilation alters arterial baroreflex control of heart rate and muscle sympathetic nerve activity. *Am J Physiol Heart Circ Physiol* 2000; **279**: H536–H541.