ORIGINAL CONTRIBUTION

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Carbon nanotubes have a deleterious effect on the nose: the first *in vitro* data*

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SUMMARY

Background: *The information currently available concerning carbon nanotubes toxicity is disturbing and conflicting. Moreover, little is known about their effect on the nasal cavities, which are the first target for nanoparticles.*

Material and method: *We investigated the cytotoxicity (50 to 0.5 µg/mL) of double-walled carbon nanotube with two independent tests (MTT, Wst-1) on normal human nasal epithelial cells after 12-day exposure (control untreated nasal cells and A549). Nasal cell differentiation function, oxidative stress, the morphological features of cells in contact with DWCNTs and the localizations of the latter were also investigated.*

Results: *Exposure revealed a dose-dependent decrease in cell metabolic activity and cell growth. In nearly all conditions, normal human nasal epithelial cells were more sensitive than malignant ones. Even with both tests, the cytotoxic threshold dose could not be accurately determined because of dye adsorption by DWCNTs. Nasal cells showed stronger cytokeratin 7 and persistent UEA-I immunostaining. Cytokeratin 19 production was increased at 25 µg/mL and mucus production was stimulated from 0.5 µg/mL. A significant increase in Reactive Oxygen Species was observed from 25 µg/mL. The cell plasma membrane showed several holes and DWCNTs were present in the cytoplasm.*

Conclusion: *DWCNTs seem to have a deleterious effect on nasal cells after 12-day exposure.*

Key words: cytotoxicity, carbon nanotube, epithelial cells, nasal cavity, nasal mucosa

INTRODUCTION

Carbon nanotubes (CNTs) could provide the nanotechnology platforms needed for enhanced intracellular delivery of very many products [(1)]. However, it is imperative that the toxicity and biocompatibility of CNTs be investigated to make them safe and useful for health applications. Despite the blossoming of research activity in the field of potential devices and applications, it is only recently that information on toxicity and biocompatibility has become available [(2)] with disturbing and conflicting results [(3-6)].

Penetration of CNTs in the body is possible by the respiratory and digestive tractus, and through the skin and connective tissue. However, CNTs are extremely aerosolized, making respiratory contamination by inhalation rather likely to occur.

The distribution and quantity of nanoparticles deposited in the respiratory tree increases with the reduction in their diameter and inspiratory airflow [(7,8)]. Moreover, their accumulation is not uniform between the nasal cavities, pharynx, larynx, trachea, bronchus, bronchioles and pulmonary alveoli [(7)]. Particles from 5 to 100 nm settle mainly in the pulmonary alveoli whereas those lower than 5 nm settle preferentially in the nasal cavities [(7)]. Oberdörster et al. showed for the same quantity that the concentration of nanoparticules per unit of area is greater than 100-fold higher in the nasal area compared to the lung because of the differences in heat-transferring surface [(9)]. These features could have important consequences on health and on the mechanisms of penetration and elimination. It is known already that many nanoparticles cross the nasal epithelial barrier to reach the meninges and the brain via the

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olfactory nerves [10-12]. To date, numerous *in vitro* and *in vivo* data are available concerning the pulmonary toxicity of CNTs but little is known about the nasal cavities [13].

The interaction between cells and CNTs is a critical issue that will determine any future biomedical application of such structures, so cell culture models used to assess cytocompatibility should be relevant and reliable. Few studies used normal and human cell models. The permanent cell lines from a wide variety of animal species and tissues, frequently of carcinoma origin [14-22], have usually been used because several authors consider these cells to have greater xenobiotic sensitivity than normal ones [23]. At present, A549 is considered as the most convenient cells type for studying respiratory immunotoxicity [24]. However, they demonstrate different degrees of sensitivity for the same material and identical culture conditions, according to the test used [5,6]. Thus, we investigated CNT toxicity on normal human nasal epithelial cells (HNEpCs) since 1) this airway epithelium of the respiratory tract is considered as a primary target when exposed to such volatile nanoparticles, 2) HNEpCs are considered as surrogates for bronchial epithelial cells [37], and 3) normal cells used *in vitro* are a more realistic cell model to mimic *in vivo* toxicity than malignant ones.

The aims of this study were to assess the cytotoxicity of Double-Walled Carbon Nanotubes (DWCNTs) on HNEpCs during 12-day exposure mimicking a sub-acute situation. Cell differentiation function, oxidative stress, the morphological features of the cells and DWCNTs localizations were also investigated.

MATERIALS AND METHODS

Synthesis and characterization of DWNTs

DWCNTs were produced by Catalytic Chemical Vapor Deposition (CCVD) according to Flahaut et al. [26]. After CCVD, the catalyst and by-products were removed by treating the sample with a concentrated aqueous HCl solution. High-resolution transmission electron microscopy showed that a typical sample consists of 80% DWCNTs, 20% single-walled nanotubes, and a few triple-walled carbon nanotubes. The diameter of the DWCNTs ranged from 0.5 to 2.5 nm for inner tubes and from 1.2 to 3.2 nm for outer tubes. The length of individual DWCNTs usually ranges from 1 to 10 μm, although bundles may be much longer (up to 100 μm at least). The DWCNTs are not functionalized. They were prepared in serum-free medium and sterilized at 120°C. Next, the suspensions were prepared by dispersing an initial concentration of 1.800 μg/mL by sonication with an ultrasonic tip (500 W) at 70 % amplitude for 5 min to ensure the whole separation of aggregates.

Cell cultures and exposure conditions

HNEpCs were purchased from Promocell® (Promocell GmbH®, Heidelberg, Germany) cultured with appropriate medium and used in passages 1 to 3. The culture medium was the airway epithelial cell growth medium provided by Promocell GmbH® (réf: C-21160). It contained 0.004 ml/ml bovine pituitary extract, 10 ng/ml epidermal growth factor, 5 lg/ml insulin, 0.5 lg/ml hydrocortisone, 0.5 lg/ml ephedrine, 6.7 ng/ml triodo-L-tyrosine, 10 lg/ml transferrin and 0.1 ng/ml retinoic acid. Under inverted light microscopy, confluent cells formed a uniform monolayer according to a previous study [27].

For cytotoxicity assays, the cell seeding density was 10.000 per cm^2 in 48-well plates. HNEpCs were submitted at sub-confluency for 12d to suspensions at 0.5-, 2.5-, 5-, 25-, 50 μg/mL of DCWNTs (corresponding to 0.15-, 0.75-, 1.5-, 7.5 and 15 μg/cm^2, respectively) prepared freshly before the experiments in the complete culture medium. The cell culture medium was renewed and changed every 2 or 3 days. Mitochondrial activity and cell growth in the presence of DWCNTs were monitored over 12 days by MTT and WST-1 assays [5,14,18,20,22]. Untreated cells were used as positive controls (100% viable) for all experiments.

To compare with the gold standard, A549 were kindly provided by Laboratoire de Pharmacologie Clinique et Expérimentale des médicaments anti-cancéreux (Paul Sabatier University, Toulouse, France) and used in passages 80 to 84. The cell seeding density was 5.000 per cm^2 in 48-well plates. They were cultured in IMDM medium supplemented with 10% fetal calf serum. Firstly, to assess adsorption of dyes onto DWCNTs, we performed control experiments in which we quantified it. A549 cultured without DWCNTs was incubated with MTT or WST-1 and the dye products (formazan) or the dye absorbances were measured in the presence or absence of DWCNTs. Next, A549 were exposed to the same DWCNTs concentrations for 12d. The same methodology of exposure was applied and untreated A549 cells were used as controls (100% viable) for all experiments.

For each cell type, results are expressed as percent of controls of two independent experiments, each carried out on 4 specimens (mean ± SD).

Cell differentiation function

Immunostaining was classically performed by immunoperoxidase technique intensified by the streptavidin-biotin complex after 12d exposure to 25, and 0.5 μg/mL of DWCNTs. HNEpCs were incubated with mouse monoclonal antibody anti-human anticytokeratin 7 (DAKO A/S Co., Glostrup, Denmark) diluted at 1:200 and rabbit polyclonal antibody anti-human UEA-I (Ulex European Agglutinin–I, DAKO A/S Co.) diluted at 1:800. Before experiments untreated HNEpC controls were systematically tested with both antibodies [28,29].

The production of mucus is a first line of defense against inhaled foreign bodies. Thus, we sought to detect the presence of glycosaminoglycans by alcian blue staining in cells. To detect acid mucins, the cells were detached from culture plastic wells by trypsin. They were transferred to Lab Tech system© glass slides and immediately fixed to avoid modification of the cell phenotype. Alcian blue staining (pH 2.5) was carried out according to a standard method [30].

Intra- and extracellular released cytokeratin 19 levels were

measured after 12 days of exposure to 25 and 0.5 µg/mL of DWCNTs from 48-well plates in triplicate using a commercial kit: ELSA-CYFRA 21-1™ (Cis bio international, Gif-sur-Yvette, France), which is a solid-phase sandwich immunoradiometric assay. These 2 concentrations were toxic and non-toxic on cells, respectively, in reference to growth curves. In the assay, precise recognition of the cytokeratin 19 fragment is performed with two monoclonal antibodies (BM 19-21 and KS 19-1). Results (within- and between-run CV being inferior to 6%) were first expressed as nanogram per milliliter for CYFRA and then normalized to intracellular protein content (evaluated by the bicinchoninic acid (BCA) protein assay (Pierce Science, Bezons, France).

Reactive Oxygen Species (ROS) assay

Oxidative stress was investigated by Reactive Oxygen Species (ROS) generation. ROS generation was determined with the 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma- Aldrich) reagent as described by L' Azou et al. [31] with some modifications. DCFH-DA is a stable, non-fluorescent molecule that is hydrolyzed by intracellular esterases to non-fluorescent 2', 7'-dichlorofluorescein (DCFH), which is rapidly oxidized in the presence of peroxides to a highly fluorescent product (dichlorofluorescein). Briefly, three days prior to each experiment, cells were seeded in 6-well plates, then incubated for 4 h or 24 h with DWCNTs at two concentrations (25 and 0.5 µg/mL) in culture medium. After incubation, cells were washed with HBSS (Hank's balanced salt solution) and incubated with 50 µM DCFH-DA for 15 min. Subsequently, cells were washed with HBSS, scraped off, lysed by sonication and centrifuged. Supernatants were collected and ROS levels were determined at excitation wavelength 488 nm and emission wavelength 520 nm using a fluorimeter (Kontrol Instrument, Eching, Germany). Data from at least triplicates are reported as fluorescence intensity percentage and expressed as mean fluorescence ratio (fluorescence of exposed cells/fluorescence of unexposed control from the same experiment) (mean ± SD).

Electron microscopy

For scanning electron microscopy (SEM), specimens at day 12 were fixed by immersion in a mixture (1:1) of a glutaraldehyde solution (2%) and a cacodylate buffer (pH 7.3). Surfaces were then washed with 0.15M cacodylate for 10 min. Samples were desiccated at room temperature and finally metalized with metal using a gold target.

For transmission electron microscopy (TEM), samples were fixed with glutaraldehyde-cacodylate buffer for 1 h at 4°C. Cells were then washed in 0.15 M cacodylate. Post-fixation with 2% (v/v) OsO4-0.3 M cacodylate was carried out for 60 min. The samples were dehydrated through a graded series of ethanol from 25 to 100%. Dehydration was completed with propylene oxide treatment and then infiltration was carried out with propylene oxide Epon (1:1). Finally, the samples were embedded in 100% fresh Epon and polymerized in a 60°C oven for 48 h. Sections of 1 µm thick were cut.

Statistical analysis

The non-parametric Mann-Whitney U-test was used to assess statistically significant differences between treated and untreated cells after cytotoxicity evaluation. The absorbance recorded from untreated cells for each period and condition was set at 100%. The same test 'U' was used to compare ELSA-CYFRA 21-1™ measurement and ROS assays between control and exposed cells. P-values less than 0.01 were considered statistically significant (StatEL 2.2 statistical software, Adscience Company, Paris, France, www.adscience.fr)

RESULTS

Cell growth

Results of cell growth are presented in Figure 1. During 12 d exposure on HNEpCs (Figure 1A), there was a clear concentration-dependent decrease in cell growth and a cumulative effect as a function of time over at least 9 days. This effect was less striking with the other test (Figure 1B) at the lowest concentration. Concerning A549 during the MTT assay (Figure 1C), we obtained a maximal effect with DWCNTs over 3 days. Thereafter there was no longer any significant inhibition. During Wst-1 (Figure 1D), the overall pattern of A549 proliferation was comparable to that in Figure 1C. Thus, in nearly all conditions, normal human nasal epithelial cells were more sensitive than A549.

However, as adsorption of dyes is known to occur onto DWCNTs, we decided to explore this phenomenon (Figure 2). Adsorption of dyes onto DWCNTs occurred with both tests at 25 and 50 µg/mL in a dose-dependent manner. High concentrations significantly lowered absorbance readings (Figure 2). However, DWCNTs seemed toxic for HNEpC because adsorption had no effect below 5 µg/mL (Figures 1, 2).

Cell differentiation function

Each untreated cell culture showed a strong immunostaining for anticytokeratin 7 and anti-UEA-I. Cytokeratin 7 immunostaining in exposed HNEpCs seemed stronger at 25 µg/mL of DWCNTs (Figure 3A-B) in comparison with unexposed HNEpCs, and was comparable at 0.5 µg/mL. UEA-I was still present in HNEpCs exposed to 25 µg/mL of DWCNTs (Figure 3C-D). Moreover, Figure 3E shows negative staining of alcian blue in control HNEpCs that became positive in cells exposed to 0.5 µg/mL of DWCNTs (Figure 3F). Total intra- and extracellular cytokeratin 19 expressed as ng per mg of protein content revealed a markedly increased production for DWCNTs only at 25 µg/mL (Figure 4). Intracellular amounts of cytokeratin 19 represented 98% of total values in all specimens. Untreated HNEpCs showed a CYFRA level of 259 ± 55 (mean ± SD) ng per mg of protein.

ROS production

Concerning oxidative stress, HNEpCs treated with 25 µg/mL of DWCNTs produced a dramatic increase in ROS induction compared with control (Figure 5). On the contrary, the non-cytotoxic 0.5 µg/mL concentration did not induce any ROS production.

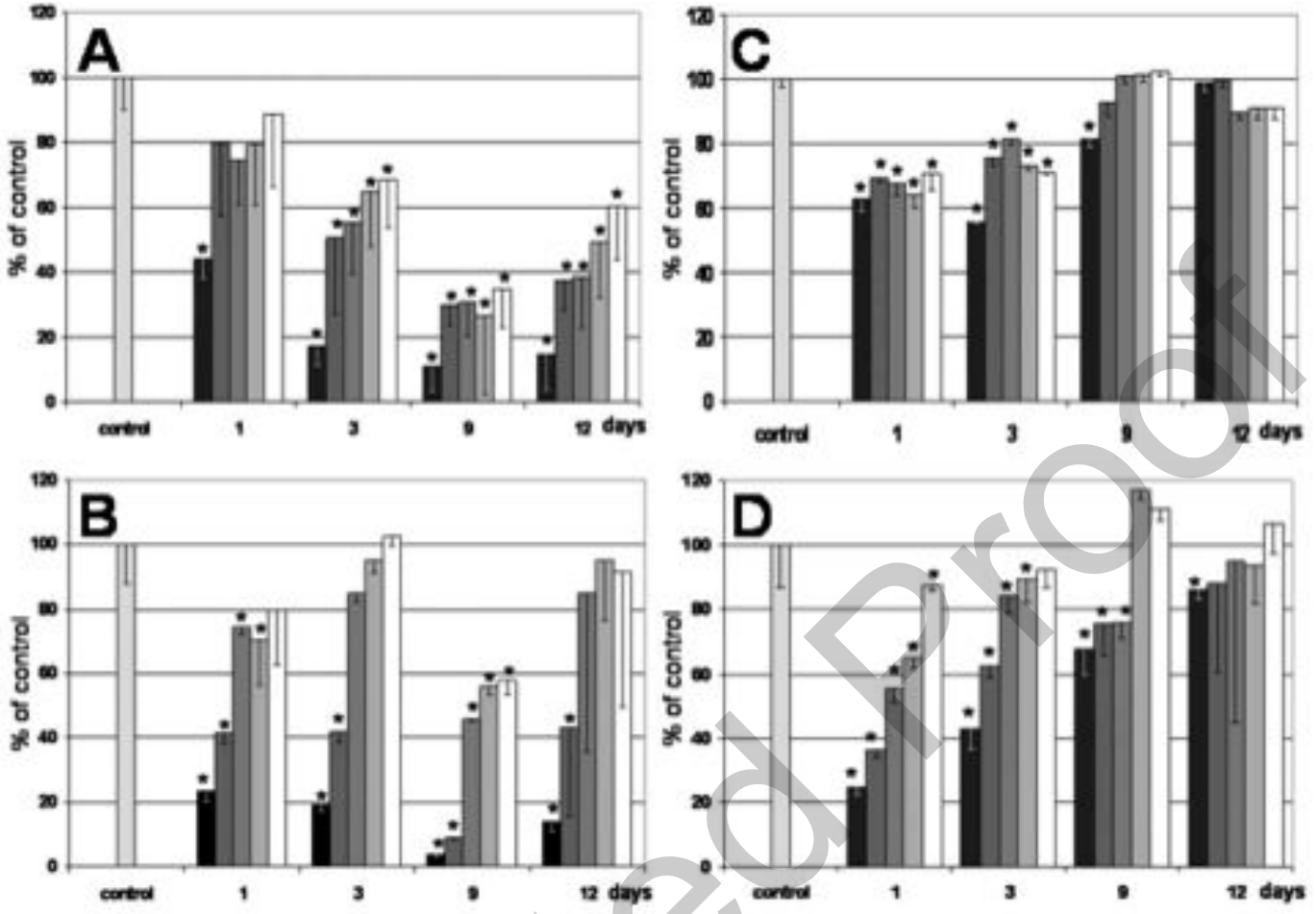

Figure 1. Results of HNEpC (A-B) and A549 growth (C-D) in the presence of 5 concentrations of DWCNTs (from 50 µg/mL (black bar) to 0.5 µg/mL (white bar)) monitored over 12 days by MTT (A-C) and Wst-1 (B-D) assays. Untreated cells were used as control set at 100 % (Lined bar). (*) refers to a significant difference from the control ($p < 0.01$).

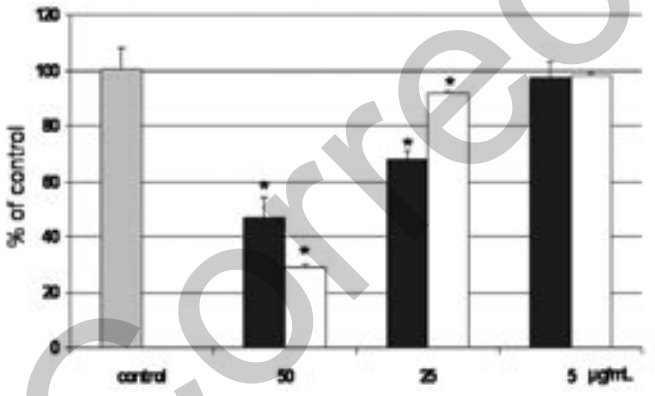

Figure 2. Control experiment to quantify dye adsorption onto DWCNTs. A549 was cultured without DWCNTs and was incubated with MTT (black bar) and Wst-1 (white bar). Next the adsorbance readings were measured in the presence and absence of DWCNTs. Untreated cells were used as control set at 100 % (Lined bar). (*) refers to a significant difference from the control ($p < 0.01$).

Electron microscopy

With regard to ultrastructural modifications, SEM revealed damaged cells in contact with aggregates, with several holes in the cell plasma membrane (Figure 6A-C). TEM showed that DWCNTs were internalized (Figure 6C-F) and that they occurred within vacuoles or cytoplasmic compartments resembling lysosomes. Higher magnifications confirmed that DWCNTs were either isolated or aggregated (Figure 6F), with a higher proportion of the latter. We never observed any DWCNTs in the cell nuclei.

DISCUSSION

It is well known that nasal cavities are a target site for air polluant-induced toxicity and carcinogenicity [7,13,32]. For this reason and because most airway epithelial research has relied heavily on commercially transformed cell lines such as A549 (alveolar adenocarcinoma), the present study of airway epithelium exposure to DWCNTs was conducted with human primary cultured nasal epithelial cells. It is well known that primary cultured cells should be used for *in vitro* analyses because they are very similar to cells *in vivo* and more sensitive to xenobiotics compared to cell lines [33,34]. Very few studies have investigated normal and malignant cells from the same epithelial origin exposed to CNTs [15,16]. The comparison of DWCNT toxicity on HNEpC and A549 seems to confirm our hypothesis.

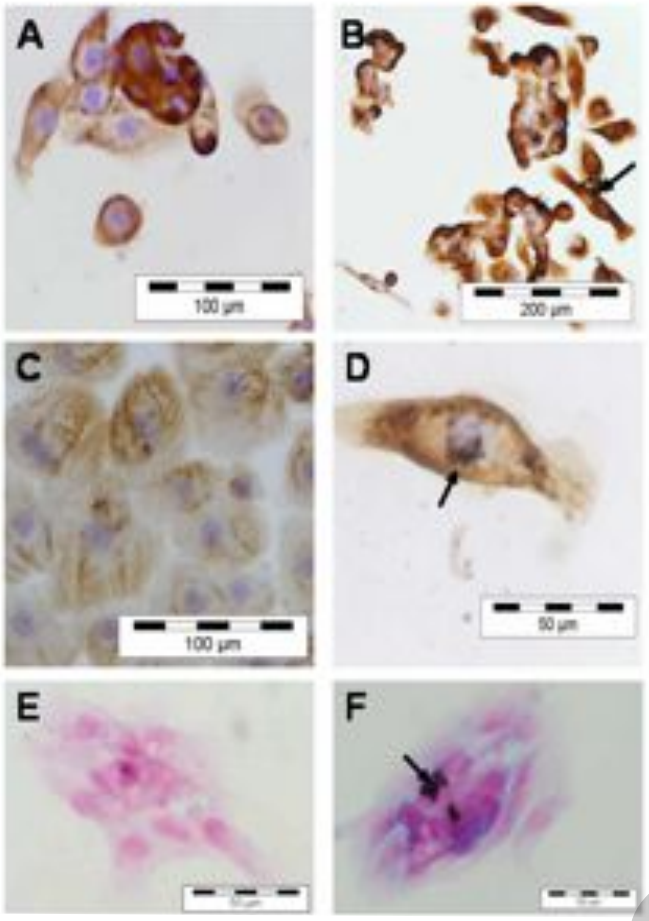

Figure 3. HNEpC characterization before and after 12d exposure with 25 µg/mL of DWCNTs: cytokeratin 7 baseline (A) and after exposure (B), expression of the glycoconjugate UEA-I before (C) and after exposure (D). Alcian blue detection in untreated HNEpCs (E) and in HNEpCs treated with 0.5 µg/mL of DWCNTs (F); black arrow shows DWCNT aggregates in contact with cells (B-D-F).

Carbon nanomaterials are known to interfere with a number of colorimetric indicator dyes [5,17,19,35]. However, metabolic activity modifications are often assessed with MTT [5,6,15,17-20] and WST-1 [5,14,19,20,36] despite certain limitations. In our study, we assessed cytotoxicity by both these independent tests as previously suggested [5,17,19] to overcome putative false positive findings. Concerning the validity of MTT and WST-1 assays, the former may provide a false positive result [5,14,17,19,20] whereas the latter may [20] or may not [5,14,22]. The latter is considered reliable [22] for assessing interactions of CNTs with dyes. However, in our study, identical adsorptive properties were found on DWCNTs for both tests. Thus, even with these two independent assays, the artifacts generated by CNTs cannot be avoided, as shown by these confounding results. It is therefore likely that DWCNTs adversely affect cell viability. However, the reliability of the tests depends on the DWCNT concentration, since cytotoxicity has to be validated in a concentration range without adsorption.

In human respiratory epithelium, cytokeratins 5, 14 and 17 are mainly expressed in the basal cells whereas cytokeratins 7, 8, 18 and 19 are localized at the surface of the bronchial, tracheal and nasal epithelium [28]. As such, they constitute a useful marker of epithelial differentiation. *In vitro* and *in vivo*, the first step of the repair process after mechanical or chemical wound involves loss of differentiation [37] and loss of immunostaining of cytokeratin 7 – 19 [36] and UEA-I [38]. Moreover, squamous metaplastic changes are considered to be an adaptive response that protects the lumen from the effects of inhaled airborne pollutants [39-41], as evidenced by the distribution of individual cytokeratins [36]. However, persistent immunostaining of cytokeratins 7 and 19 in contact with DWCNTs provided strong evidence here of the epithelial origin of the cells and lack of dedifferentiation. Moreover, the fact that UEA-I is strongly and persistently expressed in nasal epithelial cells [29] provides an additional argument for the conservation of phenotypes in contact with DWCNTs. We speculate that the maintenance of normal respiratory epithelial differentiation could be a sign that metaplasia has not begun and that a repair process following cell injury by DWCNTs is not involved.

Moreover, the first-line defense against inhaled insult impinging on and damaging the epithelium is the production of mucus [42]. Our results are consistent with goblet cell hyperplasia demonstrated by Ma-Hock et al. [43] and Pauluhn [44] in lung rat after MWCNT exposure from the first day and show the capacity of CNTs and aggregates to increase mucus production. We speculate that the absence of dedifferentiation at both concentrations combined with the production of mucus from 0.5 µg/mL or more suggests that DWCNT-induced effects are not specific to the cells that are thought to produce mucus as a response to the mechanical stimulus of nanotube aggregation.

Oxidative stress plays a key role in nanoparticle biocompatibility [14,15,19,31]. The formation of ROS in nanotube-treated cells was not evidenced with purified CNTs [14], leading the authors to the conclusion that metal traces associated with commercial nanotubes were responsible for the biological effects they found. The relation between intracellular ROS formation and the content of metal impurities is well documented [19] but our CNTs were not of commercial origin [26]. Moreover, the role of ROS in the global response of airway epithelium to particulates has already been reviewed [45] and can contribute to airway pathology. Recently, Crouzier et al. [46] showed that CNTs induced inflammation but decreased the production of ROS in rat lung. The authors attributed this decrease only to the scavenger capability of pure CNTs. However, CNTs in this study were delivered by intranasal instillation, a modality that leads to swallowing of 70 % of the dose [47], thus considerably decreasing the dose available for the entire respiratory airway, which is responsible for this lack of *in vivo* ROS production.

In our experiments, DWCNTs entered the HNEpC. The pathways of entry of SWCNTs into cells are energy-dependent phagocytosis or endocytosis and/or passive diffusion across lipid layers [21]. While we were unable in this study to determine a mechanism responsible for cellular uptake, we can rule out

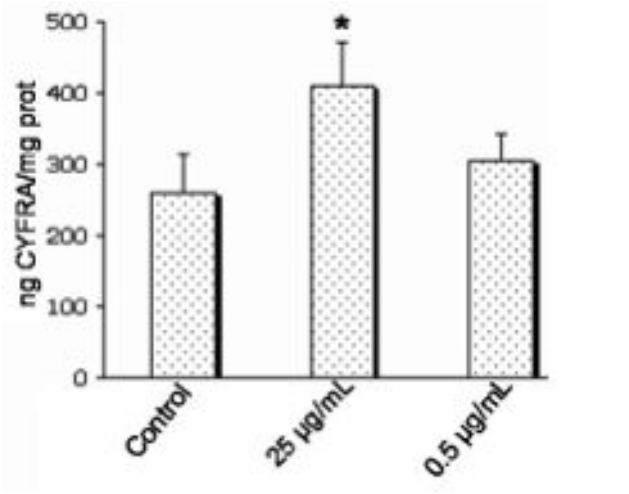

Figure 4. Cytokeratin 19 expressed as ng of CYFRA per mg of intracellular protein content (*) refers to a significant difference from the control ($p < 0.01$).

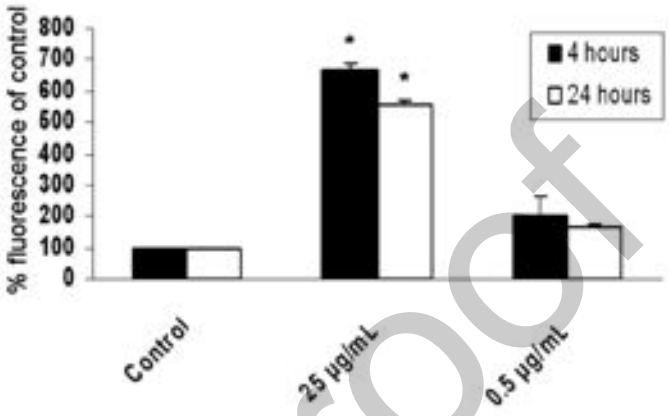

Figure 5. ROS generation in HNEpCs after 4h and 24h treatment with DWCNTs. (*) refers to a significant difference from the control ($p < 0.01$).

the phagocytosis process restricted to specialized cells such as macrophages, monocytes and neutrophils not commonly used by differentiated HNEpCs, even if the major limitation of our study remains that our cell culture model is still far from physiology.

CONCLUSION

This study aiming to mimic *in vitro* sub-acute exposure to CNTs shows that: 1) DWCNTs have a deleterious effect on normal nasal respiratory cells and that the ROS they produce may play both a direct and an indirect role in the ensuing biological responses; 2) the functional relevance of permanent malignant cell lines such as A549 is debatable since they do not represent a sufficient model because normal cells are more susceptible to CNT-induced injury; 3) a true measurement of toxicity is difficult to achieve even with WST-1. Concerning the mechanism of injury, we hypothesize that 'wound healing' following exposure of CNT is not a viable hypothesis on the basis of the absence of dedifferentiation.

AUTHORS CONTRIBUTIONS

Analysis: L de Gabory, R Bareille, R Daculsi, B L'Azou ; Data Interpretation: L de Gabory, E Flahaut, L Bordenave ; Drafting: L de Gabory, L Bordenave ; Revision: L de Gabory, E Flahaut, L Bordenave.

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DISCLOSURE AND CONFLIT OF INTEREST

The authors have no financial interests in this research project or in any of the techniques or equipment used in this study.

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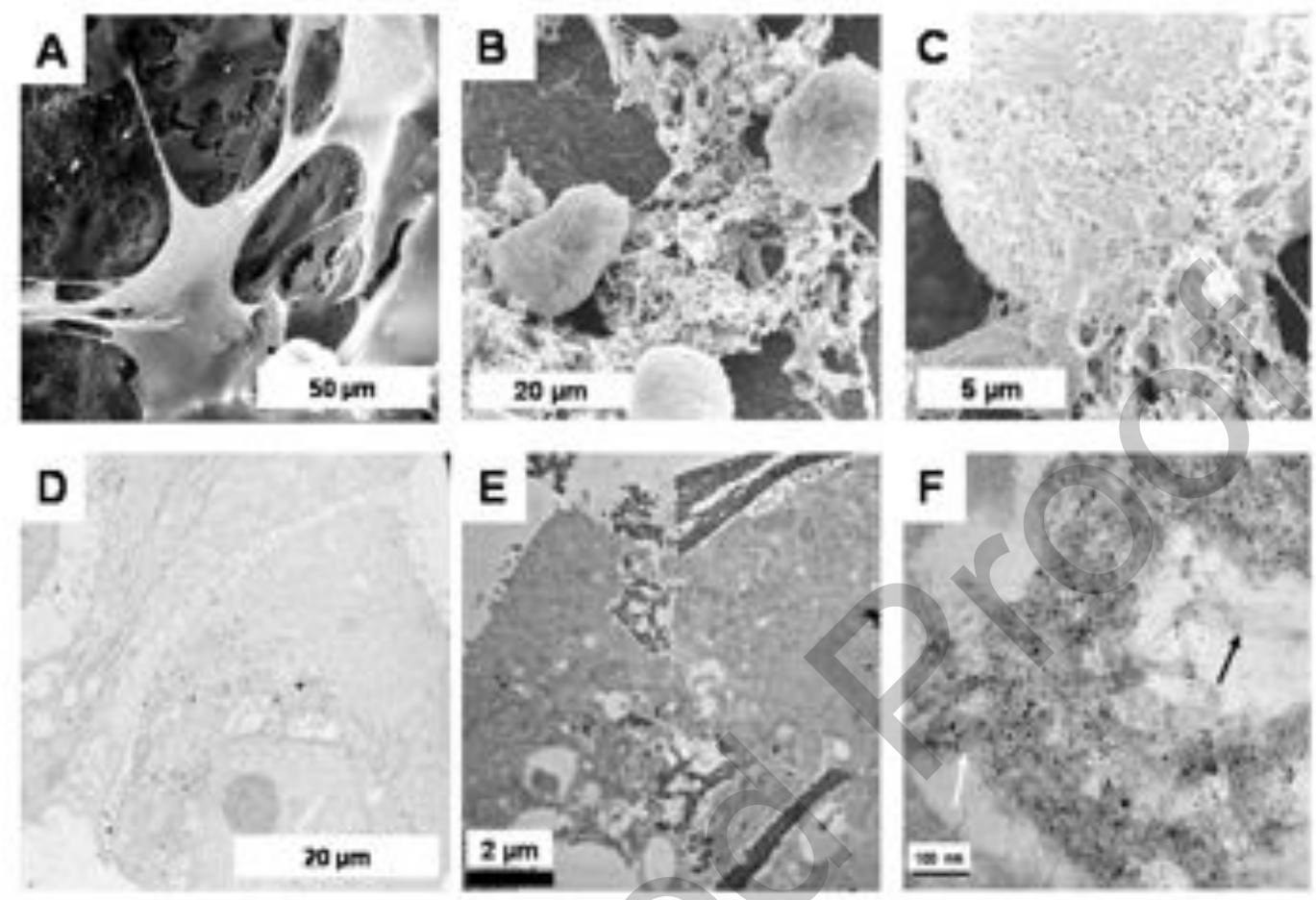

Figure 6. SEM (A-C) and TEM (D-F) magnifications of HNEpCs. (A, D) untreated cells and (B, C, E, F) treated cells over 12d with 25 µg/mL of DWCNTs: injured cells in contact with DWCNT aggregates at 1.100X (B) and higher magnification showing several holes in plasma membrane at 4.000X (C). HNEpC containing DWCNTs in lysosome-like structures at 8.200X (E) and 105.000X (F); free individual DWNTs (black arrow) and aggregates (white arrow) in endosomes and cytoplasm, black points (F) indicating remnant catalytic carbon-coated cobalt nanoparticles.

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