Multi-walled carbon nanotubes (NM401) induce ROS-mediated *HPRT* mutations in Chinese hamster lung fibroblasts

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ABSTRACT

Although there is an important set of data showing potential genotoxic effects of nanomaterials (NMs) at the DNA (comet assay) and chromosome (micronucleus test) levels, few studies have been conducted to analyze their potential mutagenic effects at gene level. We have determined the ability of multi-walled carbon nanotubes (MWCNT, NM401), to induce mutations in the *HPRT* gene in Chinese hamster lung (V79) fibroblasts. NM401, characterized in the EU NanoGenotox project, were further studied within the EU Framework Programme Seven (FP7) project NANoREG. From the proliferation assay data we select a dose-range of 0.12 to 12 µg/cm^2 At these range ~~W~~we have been able to observe significant cellular uptake of MWCNT by using transmission electron microscopy (TEM), as well as a concentration-dependent induction of intracellular reactive oxygen species. In addition, a clear concentration-dependent increase in the induction of *HPRT* mutations was also observed. Data support a potential genotoxic/ carcinogenic risk associated with MWCNT exposure.

Key words: Multi-walled carbon nanotubes; ROS; V79 cells; Genotoxicity; *HPRT* gene.

1. Introduction

Multi-walled (MW) carbon nanotubes (CNT) consist of several concentric graphene sheets with high length *vs* thickness ratio. In general, both single- (SWCNT) and multi-walled carbon nanotubes (MWCNT) possess unique physico-chemical properties with potential use in many fields (Mundra et al., 2014; Zhang et al., 2014;Titirici et al., 2015).The large number of MWCNT applications implies that their presence in the environment is dramatically increasing. This has caused new environmental and health concerns, particularly since CNT exposure has been compared to asbestos, a well-known agent inducing mesothelioma and lung cancer (Takagi et al., 2012).

Among the potential health risks associated with MWCNT exposure, those involving damage to DNA are among the most critical. Different *in vitro* studies have shown that exposure to MWCNT can be associated with the induction of primary DNA damage (Ghosh et al., 2011; Cavallo et al., 2012, Ursini et al., 2012; Linderberg et al., 2013), as well as chromosome damage (Sargent et al., 2012; Kato et al., 2013; Siegrist et al., 2914).

Due to the existence of different mechanisms inducing genotoxicity, a battery of assays testing for different genetic endpoints is required. The current test guidelines include, among the assays to be considered, an *in vitro* mammalian gene mutation test such as the forward gene mutation assay in the X chromosome~~sex~~-linked hypoxanthine phosphoribosyl transferase locus *HPRT* (OECD, Guideline 476). In this assay mutant cells are selected by incubation with a purine analogue that is toxic to normal cells but not to mutants. The *HPRT* mutation assay has been already successfully applied to the evaluation of different nanomaterials (NMs) (Doak et al., 2012). Thus, positive effects were obtained using TiO_2 NMs (Wang et al., 2007; Chen et al., 2014) and silver NMs (Huk et al., 2014), but negative findings were reported for synthetic amorphous silica NMs (Guichard et al., 2015). Interestingly, negative results were obtained with TiO_2 NMs in a long-term (60 days) exposure experiment. In this case CHO cells appear to adapt to

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the chronic exposure to nano-TiO_2 and detoxify an excess of reactive oxygen species (ROS), possibly through up-regulation of super oxide dismutase (SOD), in addition to reducing particle uptake (Wang et al., 2011). With regard to studies using MWCNT to induce *HPRT* mutations, a previous study was reported (Asakura et al., 2010) where no effects were observed in the CHL/IU cell line; however, a positive effect was observed in lymphoblastoid cells, but using SWCNT (Manshian et al., 2013). It is perhaps relevant to point out that germ-line *HPRT* mutations in humans lead to a sex-linked human neurological disorder called the Lesch–Nyhan syndrome, and that biomonitoring of human populations has shown that somatic mutations at the *HPRT* gene are linked to genotoxic/carcinogenic risk (Albertini, 2001).

In this context our work aims to investigate the ability of MWCNT to induce mutations in the *HPRT* gene. Cellular uptake of MWCNT was assessed by transmission electronic microscopy (TEM) and the induction of ROS, as a potential mechanism of action, was also determined.

2. Material and methods

2.1. Cell cultures

Chinese hamster lung fibroblast cells (V79) were cultured in 75 cm^2 flasks in DMEM low glucose medium (Sigma), with 10% FBS, 1% penicillin–streptomycin and L-glutamine (Sigma) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Nanomaterial characterization, dispersion and cell exposure

The selected MWCNT (NM401) wasere obtained from the EU Joint Research Centre (Ispra, Italy). This material was well characterized in the EU Nanogenotox project (2012), with transmission electron microscopy (TEM) showing diameter 64.2 ± 34.5 nm and length 4048 ± 2371 nm with a DLS (Dynamic Light Scattering) Zeta size in 0.05% BSA medium of 710 ± 17 nm. It also contains a small amount of impurities accounting for 0.11% of the total weight including Al, Mg, Na, Cr, Fe and Co traces (Nanogenotox, 2014). Further confirmatory studies were carried out by us using TEM, to obtain size and morphology, on a JEOL JEM-2011 instrument.

For dispersion procedures the Nanogenotox protocol (2011) was used. Briefly, MWCNT were pre-wetted in 0.5% absolute ethanol and afterwards dispersed in 0.05% bovine serum albumin (BSA) in MilliQ water and sonicated for 16 min to obtain a stock dispersion of 2.56 mg/mL.

2.3. Cellular uptake detection by TEM

V79 cells, unexposed and exposed to 12 µg/cm^2 NM401 for 24 h, were fixed in 2.5% (v/v) glutaraldehyde (EM grade, Merck, Darmstadt, Germany) and 2% (w/v) paraformaldehyde (EMS, Hatfield, PA) in 0.1 M cacodylate buffer (PB, Sigma-Aldrich, Steinheim, Germany), pH 7.4. Samples were first post-fixed with osmium, dehydrated in acetone, later embedded in Epon, and finally polymerized at 60 ºC and cut with an ultramicrotome. Ultrathin sections were placed on copper grids and contrasted with uranyl acetate and Reynolds lead citrate solutions and then observed using a Jeol 1400

(Jeol LTD, Tokyo, Japan) TEM equipped with a CCD GATAN ES1000W Erlangshen camera (Annangi et al., 2015). The presence of MWCNT inside the cells was determined by visualizing different TEM images.

2.4. Intracellular ROS analysis by flow cytometry

The generation of intracellular reactive ROS was determined by flow cytometry using the 6-carboxy-2,7´-dichlorodihydro-fluorescein diacetate (DCFH-DA) assay (Toduka et al., 2012). Treated and untreated cells were seeded in triplicate in 6-well plates at a density of 1 x 10^5 cells/well. Treatments were carried out 24 h after seeding. After 24 h of NM401 exposure, cells were washed twice with PBS and incubated in 5 µM DCFH-DA in serum-free DMEM medium for 30 min at 37 ºC. The presence of intracellular ROS converts the non-fluorescent DCFH-DA to its fluorescent form, measured using fluorescence-activated cell sorting (FACS; Calibur). Flow cytometry used excitation and emission wavelengths of 480 nm and 530 nm, respectively. Data were analyzed with the software Flowjo Ver. 7.6.5.

2.5. Proliferation assay - relative cell growth activity (RGA)

V79 cells were seeded on 12-well plates (1 × 10^5 cells per well) and incubated at 37 °C. After 24 h, cells were exposed to NM401 for 24, 48 and 72 h at concentrations ranging from 0.12 to 75 µg/cm^2. After exposure, medium was removed; cells were washed with PBS, trypsinized and re-suspended in 1 mL medium. Ten µL of the cell suspension was mixed with 10 µL 0.4% trypan blue (Invitrogen) and the percentages of living cells (unstained) and stained cells with damaged membranes were measured using a Countess™ Automated Cell Counter (Invitrogen). RGA was calculated as already published (Huk et al., 2014).

2.6. Plating efficiency (PE)

To determine cytotoxicity, V79 cells exposed to NM401 for 24 h were washed, trypsinised and counted as described above. ~~50~~ Fifty cells per well were inoculated in 6-well plates (1 plate for each concentration tested) and left in an incubator at 37 °C for 7 days. Cells were then stained with 1% methylene blue (Sigma) and the number of colonies was counted manually. PE was calculated according to the follow formula:

$$PE\ (\%) = \frac{number\ of\ colonies\ in\ exposed\ cultures}{number\ of\ colonies\ in\ unexposed\ cultures} \times 100\ \%$$

2.7. HPRT gene mutation test

The mammalian *in vitro HPRT* gene mutation test was carried out according to OECD guidelines (OECD 476). V79 cells were seeded on 6-well plates (1×10^5 cells per well) and incubated at 37 °C. After 24 h, the cells were exposed to NM401 for 24 h, at concentrations ranging from 0.12–12 μg/cm^2. After exposure, the medium was removed, and cells were washed, trypsinized and re-suspended in medium. Cells were seeded in 100 mm Petri dishes (3×10^5 cells/Petri dish, 3 dishes per sample to achieve approximately 10^6 cells per sample), and cultivated in culture medium for an additional 8 days period. Cells were harvested twice for detecting mutants at days 6 and 8 after the treatment. Cells were inoculated in 100 mm Petri dishes and grown in selective medium containing 6-thioguanine (5 μg/mL, Sigma) for 10 days to form colonies. For each sample two replicas were used. Mutant colonies were stained with 1% methylene blue and counted manually. Only colonies with a minimum of 50 cells were considered.

The number of surviving cells was assessed by PE assay as described above in each of the two harvests: treated and untreated cells were plated into 6-well plates (50 cells per well, 1 plate for each sample) and incubated at 37 °C for 7 days to form colonies. ~~Cells were stained with 1% methylene blue and colonies were counted manually~~. Viability was determined at the time of each mutation harvest (i.e. 6 and 8 days after exposure) and calculated on the basis of the number of colonies versus the number of inoculated

cells and mutant frequency was determined as described previously (Huk et al., 2014). Methyl methanesulfonate (MMS, 0.1 mM, 3 h) (Sigma) was used as positive control.

2.8. Statistical analysis

All data are expressed as the mean ± SD of two independent experiments. Statistical comparison was made using the non-parametric test Mann-Whitney. Statistical analyses were performed using Prism 5.0 (GraphPad Software). Differences with $P<0.05$ were considered statistically significant.

3. Results

3.1. MWCNT (NM401) characterization

In addition to the thorough characterization of the NM401 MWCNT carried out as part of the EU NanoGenotox project, we performed a TEM analysis to determine the NM401 aspect, mainly with regard to their length. In Figure 1 we show two representative pictures; the observed lengths of around 4 µm are in agreement with the data previously reported by the NanoGenotox EU project (2012).

3.2. Cellular uptake and intracellular ROS production

We used TEM to determine the internalization of NM401 MWCNT in V79 cells. As shown in Figure 2, V79 cells were able to take up the selected NMs. Different images were recorded and in about 80% of the cases cells contained significant amounts of NM401 inside the cells; most were seen in endosomes, but none in nuclei.

We further evaluated whether exposure of V79 cells to NM401 resulted in generation of intracellular ROS. Using flow cytometry we determined the conversion of the non-fluorescent DCFH-DA to fluorescent oxidized DCF as an indication of ROS production. Figure 3 shows a clear increase in ROS levels with increasing concentration (from 0.12 to 12 µg/cm^2) of NM401.

3.3. Cytotoxic effects of NM401 on V79 cells

The cytotoxicity of NM401 was measured by determining the RGA and the PE. RGA was determined as the ratio of the number of living cells at 24, 48 and 72 h in exposed versus unexposed cultures. Figure 4 shows that there was a concentration- and time-dependent increase in toxicity,

PE was determined as the ratio of the number of colonies in exposed cultures versus unexposed. Exposure lasted for 24 h and colonies were determined after 7 days of growing. Figure 5 shows a significant decrease in PE at the highest concentration of NM401. From the range of doses initially selected (0.12 to 75 µg/cm^2), 15 and 75 µg/cm^2

showed to be be highly toxic and, as consequence, all the other studies were carried out using a dose-range covering from 0.12 to 12 μg/cm^2.

3.4. Mutagenic effect of NM401 measured by the HPRT gene mutation assay

The mutagenic potential of NM401 was assessed in V79 cells in two different experiments for each harvest point. Figure 6 shows the increase in *HPRT* mutant frequency with NM401 concentration, to a level of about 50 mutants per 10^6 viable cells – approaching the level of 92 per 10^6 viable cells seen with the positive control (0.1 mM MMS)

4. Discussion

This study demonstrates that NM401 MWCNT is able to be internalized by V79 cells inducing significant increases in the levels of intracellular ROS that are associated with induction of mutants at the *HPRT* locus.

Increasing levels of ROS have been considered as a major component in toxicity associated with exposure to xenobiotics. In fact, oxidative damage has been proposed as a common mechanism of action of NMs (Møller et al., 2013). Different studies have demonstrated the ability of MWCNT to induce ROS in different cell lines. In the human lung cancer (A549) cells significant increases in the induction of ROS have been reported under different exposure conditions (Srivastava et al., 2011; Ju et al., 2013; Visalli et al., 2015). Similarly, ROS induction was seen in murine macrophages (Jian et al., 2013; Zhang et al., 2015). In addition to these *in vitro* studies, ROS production after MWCNT exposure has also been observed in *in vivo* models such as Swiss-Webster mice where ROS induction was detected in liver (Patlolla et al., 2011), and in C57BL/6 mouse where ROS were observed in lungs (Poulsen et al., 2015). Our results obtained in V79 cells support the view that NM401 exerts its toxic effects via the induction of oxidative stress.

Although the genotoxic and mutagenic potential of NMs have been evaluated using different approaches, methods to determine the mutagenic potential at defined loci have hardly been used. In particular, scant data exist of studies using the *HPRT* locus to determin~~eing~~ the mutagenic potential of NMs. The *HPRT* forward gene mutation assay allows quantification of base substitutions, amplifications, or small deletions. This assay has been used to determine the mutagenicity of titanium dioxide NMs in cultured human lymphoblastoid cells (Wang et al., 2007) and in V79 cells (Chen et al., 2014). Two other NMs have also been evaluated by using the *HPRT* assay; silver NMs were shown to be mutagenic in V79 cells, this effect depending of their size (Huk et al., 2014), while amorphous silica NMs were evaluated for both *HPRT* mutants and ROS production in V79 cells, with negative results (Guichard et al., 2015).

Only two studies testing the mutagenicity of CNT in the *HPRT* assay have been published until now. The first one (Asakura et al., 2010) was carried out in Chinese hamster lung (CHL/IU) cells using MWCNT with a width of 88 nm and average length of 5 µm. They were unable to induce mutations at the *HPRT* locus, although they did induce micronuclei. However, ROS production was not studied. Toxicity of MWCNT was associated with the dispersion protocol used. Fibers were incompletely internalized in the cells and localized in the cytoplasm. According to the positive results in the micronucleus assay, the authors proposed that the genotoxicity of MWCNT was related to disruption of cell division mechanisms. In summary, the observed differences with regard to the study of Asakura et al. (2010) can be attributed to both differences in the capacity of the cell line to uptake the MWCNT, or in their physico-chemical characteristics. The lack of information on the ability of the MWCNT used by Asakura et al. (2010) to produce ROS is crucial taking into account our proposal that HPRT mutant induction is mediated by ROS induction.

The second study, carried out with SWCNT detected induction of *HPRT* mutants in lymphoblastoid (MCL-5) cells (Manshian et al., 2013). Mutagenicity was associated with size; only SWCNT of 1-2 nm diameter and 1-3 µm length had a positive effect. SWCNT of this size produced significant levels of ROS and the most striking degree of transcriptional alterations affecting different oxidative stress-related genes. No mutagenic effect was observed for shorter (400-800 nm) and longer (5-30 µm) SWCNT fibers but these SWCNT also induced ROS, suggesting that ROS production alone was not sufficient to induce mutagenicity. Thus, the secondary structure that the CNT take on under experimental conditions is likely to be of importance in its mutagenic potential (Manshian et al., 2013). Interestingly, our NM has a length of about 4 µm similar to that reported as mutagenic by Manshian et al. This would support our view suggesting that mutagenicity is associated with ROS induction, and in addition with length. Nevertheless, to determine the influence of ROS induction, separately from the length effect, studies using antioxidants to reduce ROS levels would be needed.

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The *HPRT* gene mutation assay has been widely used in human biomonitoring and is seen as a biomarker of genotoxic/carcinogenic risk (Albertini, 2001). The mammalian gene mutation test is a surrogate *in vitro* marker used in cancer risk assessment together with the micronucleus assay, especially for NMs. It has great advantage compared to the bacterial Ames test. The Ames test has serious limitations for testing NMs due to the small size of bacteria and no or limited uptake of NMs through the bacterial cell wall (Magdolenova et al., 2014).

Due to their fiber-like similarities with asbestos (Poland et al., 2008) several studies have been conducted to investigate the carcinogenic potential of MWCNT. For example, rats exposed to MWCNT showed a clear association between cytotoxicity, inflammation, genotoxicity and carcinogenicity related to the diameter of the MWCNT used (Nagai et al., 2011). In addition, in *p53*-heterozygous mice MWCNT exposure induced significant incidence of mesothelioma. These results show a clear link between exposure to MWCNT, induction of oxidative stress, mutation induction and carcinogenesis. Thus mammalian gene mutation seems important when assessing the potential genotoxic/carcinogenic risk of NMs including MWCNT exposure. Our results also demonstrate the asbestos-like mechanism of action of MWCNT associated with length around 4 μm. However, additional studies are needed to confirm ROS- and length-mediated carcinogenicity of MWCNT, using CNT of the same composition and diameter but different length.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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LEGEND FIGURES

Figure 1. Representative TEM NM401-MWCNT images from a 1/10 dilution in water obtained from the stock solution. Nanotubes of different length and diameter can be observed. (A) 10000x, scale bar 2 µm, (B) 25000x, scale bar 1 µm.

Figure 2. Representative TEM figures of multi-walled carbon nanotubes (MWCNT, NM401) uptake by Chinese hamster lung fibroblast (V79) cells. A) Untreated V79 cell. B) Exposed cells where NM401 are~~is~~ observed in the cytoplasm. C) Detail of figure B at higher magnification.

Figure 3. A) FACS charts of non-exposed and exposed cells.The upper and lower right hand quadrant of each plot represents DCFH-DA fluorescent~~ce~~ cells due to ROS generation whereas the events shown in upper and lower left hand quadrant are non-fluorescent cells. B) Graphic presentation of the percentages of fluorescent cells as indicative of intracellular ROS production. As observed a direct dose-response effect is detected. Significant difference from unexposed cells (*P< 0.05, **P< 0.01).(~~***P < 0.0001~~).

Figure 4. Cytotoxic effects of NM401 measured by the relative growth activity (RGA %) in V79 cells. RGA experiments were carried out after 24, 48 and 72 h of exposure. Cells were treated with 5 concentrations (µg/cm^2) of MWCNT NM401 and cell number was counted in each time point immediately following staining. The data are expressed as mean ± SD of two independent experiments. *a statistically significant (P< 0.05) difference from the unexposed (control) cells.

Figure 5. Cytotoxic effects of NM401 measured by the plating efiiciency (PE %) in V79 cells. Experiments were carried out after 24 h of exposure. Cells were treated with 5 concentrations (µg/cm^2) of MWCNT NM401 for 24 h. Immediately after the exposure 50 cells per dish were inoculated and the number of cell clones was calculated after 10 days of incubation. Cytotoxicity of MMS has not been observed (PE = 97.99 ± 6.14%).

Figure 6. Induction of *HPRT* gene mutants after the exposure of V79 cells to different concentrations of NM401 for 24 h. Results are from two different harvests with two replicates. The mutant frequencies (x10^6) are expressed as the mean ± SD of two independent harvests per experiment. *HPRT* gene mutant frequency in treated cells with

positive control MMS (0.1 mM, 3 h), which gave 92.10 ± 29.70 *HPRT* gene mutants. Significant difference from unexposed cells (**P*< 0.05, ***P*< 0.01).

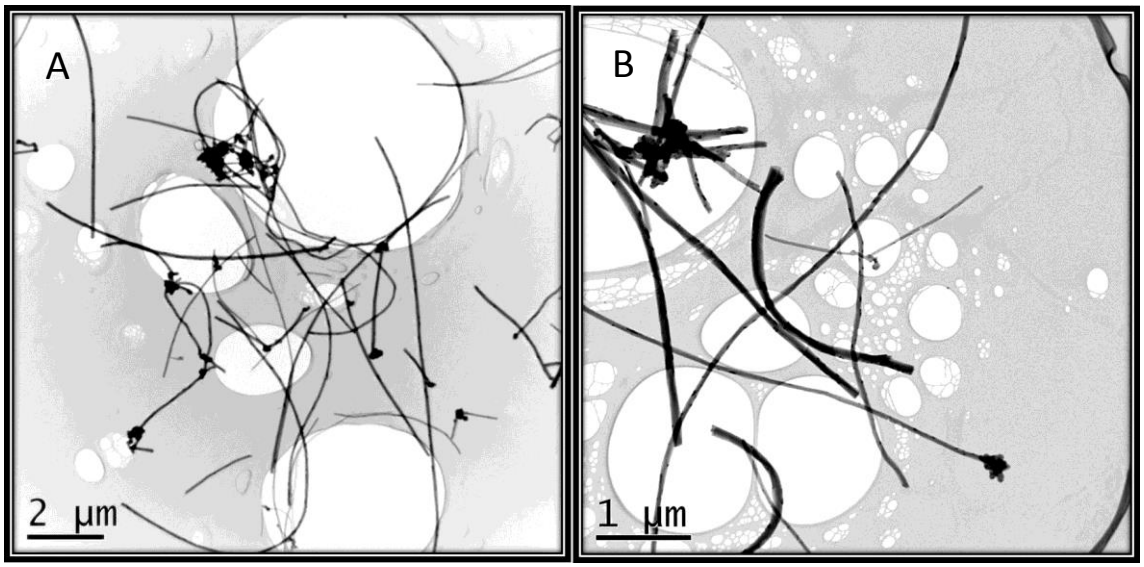


Figure 1

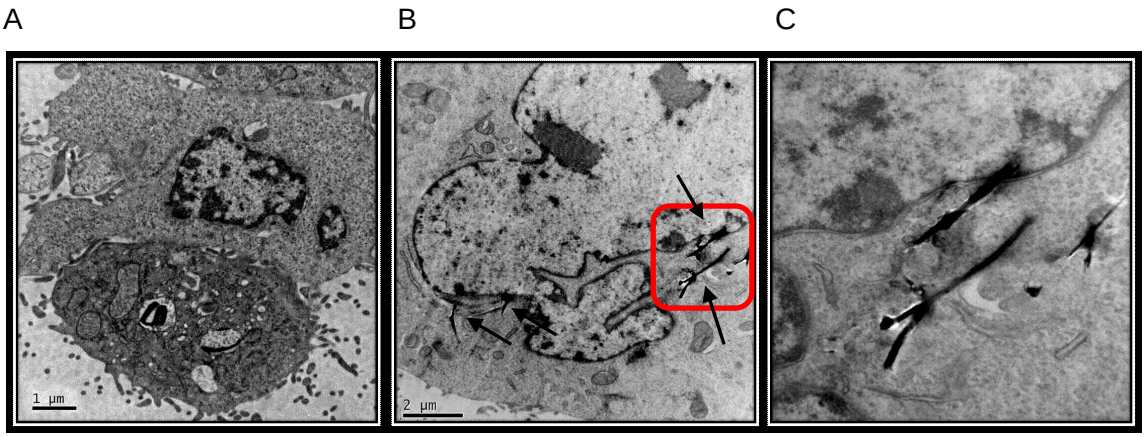
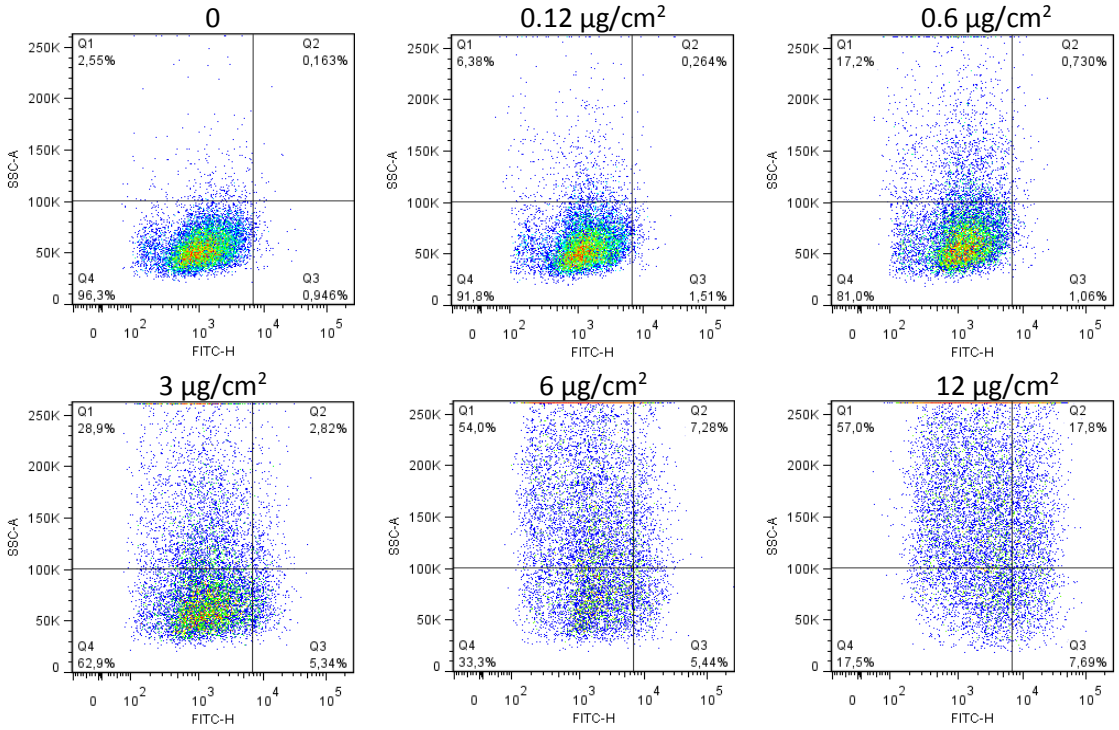


Figure 2

A



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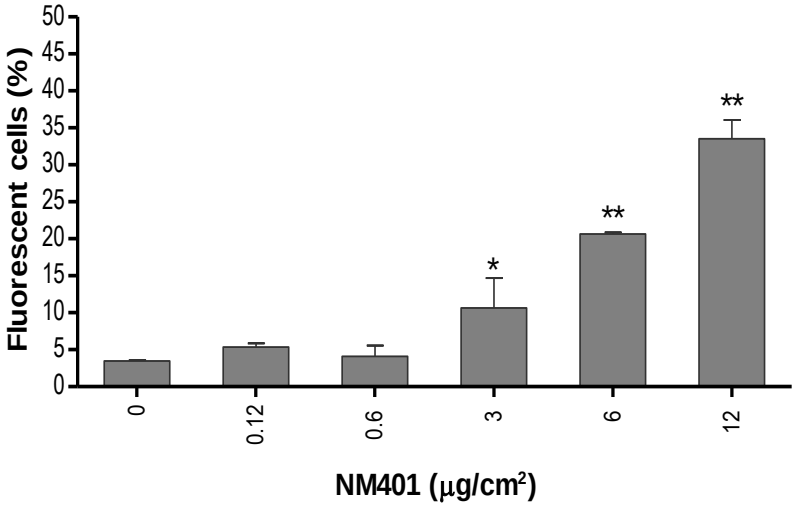

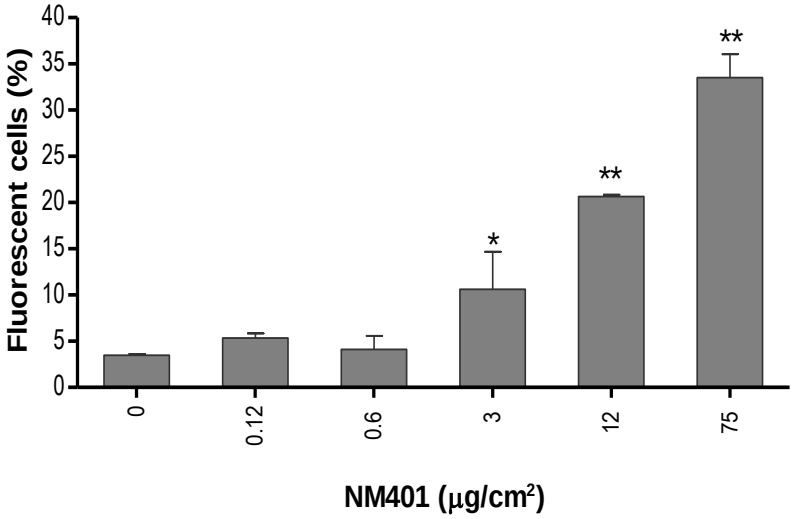

Figure 3

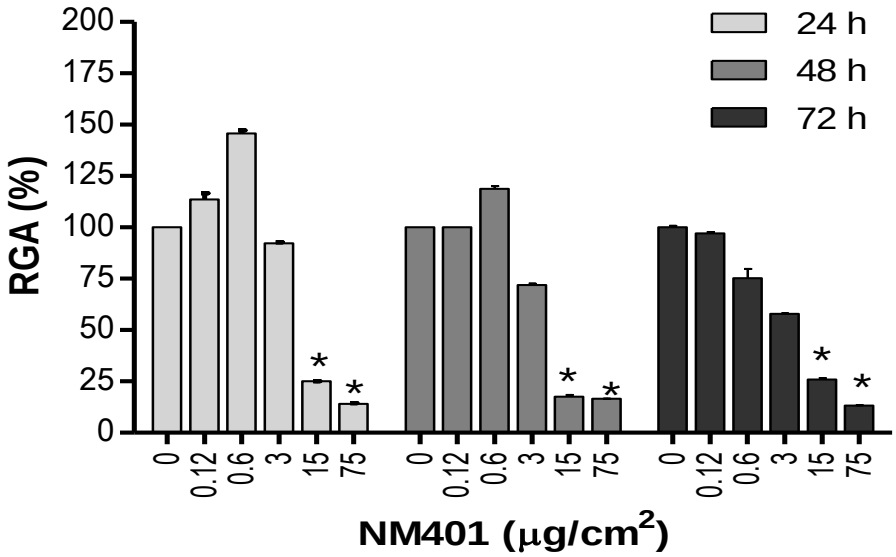

Figure 4

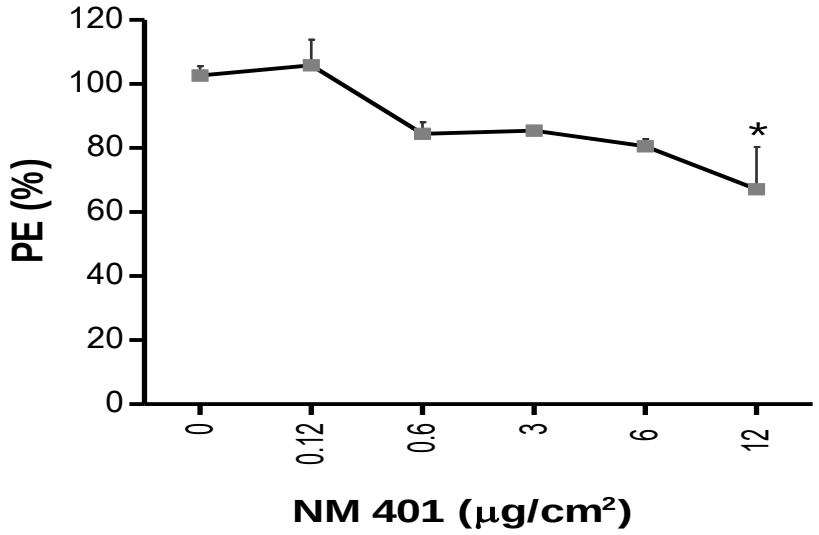

Figure 5.

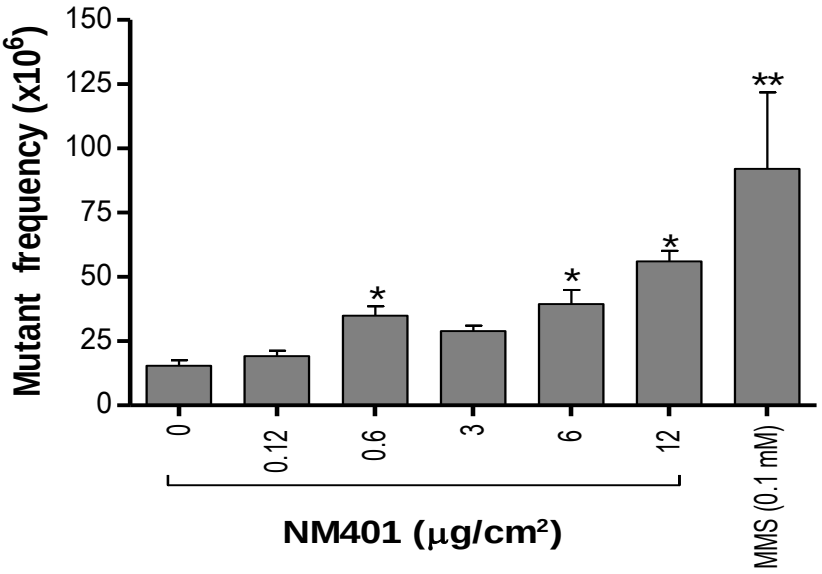

Figure 6.

This is a postprint version of: Rubio, L., El Yamani, N., Kazimirova, A., Dusinska, M., Marcos, R. (2016). Multi-walled carbon nanotubes (NM401) induce ROS-mediated HPRT mutations in Chinese hamster lung fibroblasts. Environmental Research, 146, 185-190. Published version: https://doi.org/10.1016/j.envres.2016.01.004