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Lack of mutagenic effect by multi-walled functionalized carbon nanotubes in the somatic cells of *Drosophila melanogaster*

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ABSTRACT

Carbon nanotubes (CNTs) are formed by rolling up a single graphite sheet into a tube. Among the different types of CNTs, the multi-walled carbon nanotubes (MWCNTs) comprise a set of concentric nanotubes with perfect structures. Several uses for MWCNTs have been suggested to be included in biological applications such as manufacturing of biosensors, carriers of drugs. However, before these materials can be put on the market, it is necessary to know their genotoxic effects. Thus, this study aims to evaluate the mutagenicity of multi-walled carbon nanotubes (MWCNTs) functionalized in somatic cells of *Drosophila melanogaster*, using the somatic mutation and recombination test (SMART). This assay detects the loss of heterozygosity of marker genes expressed phenotypically on the wings of the fly. Larvae of three days were used, resulting from ST cross, with basal levels of the cytochrome P450 and larvae of high metabolic bioactivity capacity (HB cross). They were treated with different concentrations of MWCNTs functionalized. The MH descendants, analyzed in both ST and HB crosses, had no significant effects on the frequency of mutant. Based on the results and on the experimental conditions mentioned in this study, it was concluded that MWCNTs were not mutagenic in *D. melanogaster*.

1. Introduction

The pharmaceutical industry has great interest in the use of carbon nanotubes (CNTs) as molecular carriers (proteins, nucleic acids and other bioactive molecules) with high selectivity for the delivery of drugs. The CNTs may act as effective vehicles for therapeutic agents in the treatment of various diseases (Mishra et al., 2010). Although nanotechnology has grown rapidly and nanoparticles may revolutionize the treatment of various diseases (Parveen et al., 2012), it is necessary that any genotoxic effects of these materials be identified before they can be used as carriers of drugs and vaccines (Kisin et al., 2011). There are three main types of CNTs: single-walled carbon nanotubes (SWNTs), double-walled carbon nanotubes (DWCNTs) and multi-walled carbon nanotubes (MWNTs). We believe that the MWNTs are more important because of their relatively low production costs and availability in large quantities is more attractive than SWNTs. In patients with cancer, MWNTs have potential roles in delivering pharmacologic agents, as diagnostic imaging agents, DNA, silent interfering RNA, oligonucleotides and proteins to detect or treat cancerous cells.

Currently, numerous studies have demonstrated that single (SWCNT), double (DWCNT) or multiple walled (MWCNT) nanotubes can induce DNA damage with the formation of micronuclei, disruption of the mitotic spindle, and induction of polyploidy (Kisin et al., 2011). Tests for genotoxicity, cytotoxicity and apoptosis performed on human fibroblasts using MWCNT (40, 200, 400 mg/μL), have indicated a dose-dependent toxicity, inducing DNA damage and programmed cell death (Patlolla et al., 2010). Our studies were done with MWCNT, however, there is divergences in the literature with respect to mutagenicity, toxicity, of various forms of carbon nanotubes. Studies conducted by Granato et al. (2010), which tested the cell viability of fibroblasts (L-929), verified that the catalysts (iron or nickel) used in the construction of SWCNTs interfere with cell growth. Results obtained in another study using single walled carbon nanotubes and graphite nanofibers (3.8–380 μg/mL), evaluated by the comet test, also demonstrated a correlation between genotoxicity and the activity of metal catalysts present in the material (Lindberg

Abbreviations: BH, balanced heterozygote descendants; DXR, doxorubicin; HB, high bioactivation cross; CNTs, carbon nanotubes; SWCNTs, single walled carbon nanotubes; DWCNTs, double walled carbon nanotubes; MWCNT, multi-walled carbon nanotubes; MH, marked trans heterozygous descendants; SMART, somatic mutation and recombination test; ST, standard cross.

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et al., 2009). The toxicity and genotoxicity mechanisms of the CNTs have not yet been fully clarified and there is a need for more research in this area (Franchi et al., 2011), but evidence suggests points of similarity between CNTs and asbestos fibers, due to the generation of reactive oxygen species and oxidative stress (Sanchez et al., 2009). Although cell viability assays performed by a few researchers have detected that SWCNT samples containing residual iron have a high cytotoxic potential (Franchi et al., 2011), the MWCNT samples used in the present study catalyzed with C, O and Fe demonstrated no cytotoxicity according to the test applied. This finding may be associated with the high purity levels of the nanotubes used in our study: only 1.5% from iron oxide and the other 98.5% from carbon. Work conducted in relation to toxicity *in vitro* SWCNTs demonstrated that exposure of cultured human cells HaCat (keratinocytes) and bronchial epithelial cells, to this biomaterial, resulted in the generation of reactive oxygen species (ROS), lipid peroxidation, oxidative stress, mitochondrial dysfunction and changes in cell morphology (Shvedova et al., 2003). On the other hand, Petersen et al. (2013) suggest that SWCNTs can help protect DNA molecules from oxidative damage. According to Ema et al. (2012) MWCNTs had no genotoxic activity in the *in vivo* micronucleus test, by the lack of micronuclei or chromosomal damage in mouse bone marrow erythrocytes at the maximum dosing limit.

Genetic toxicology is an important area of the science that studies the genotoxic/mutagenic properties of agents (chemical, physical and biological) to which the organisms are exposed, using various assays to assess the damage that these may cause to the DNA, in the presence or absence of mass metabolism systems. These assays include the SMART (Somatic Mutation and Recombination Test), developed by Graf et al. (1984). The use of SMART on *Drosophila melanogaster* wings can detect a wide spectrum of genetic abnormalities such as mutation, deletion and recombination (Graf et al., 1984). The test is based on the fact that during the early embryonic development of *D. melanogaster*, groups of cells proliferate mitotically until they become differentiated into body structures of the adult fly. If there is a genetic alteration in an imaginal disk, a clone of mutant cells will be formed and it will be detected as a spot on the wings of the mutant adult fly (Guzmán-Ricón and Graf, 1995). The analysis of these spots determines the phenotypic expression of the marker genes *flr3* or *mwh*, responsible for changes in the shape of the hairs or trichomes (Graf et al., 1984).

Given the variety of applications and the enormous potential of these nanomaterials, there are also great challenges to be faced. These include analyzing and comparing the potential toxicity and ability to induce DNA damage of the different nanoparticles. Extensive investigations are ongoing but no consensus has been reached as to the true risks because of controversial results, specifically related to the methodologies applied, the doses and the complexity of nanomaterials used. As a result, the present research is relevant in that it contributes to the process of research in this area since it had, as its objective, an evaluation of multi-wall nanotubes, functionalized, in terms of their mutagenic potential in somatic cells of *D. melanogaster*, *an vivo* eukaryotic assay system that detects a broad spectrum of genetic alterations.

2. Materials and methods

2.1. Chemicals

Flas

ks of 50 mg of doxorubicin hydrochloride (DXR) or "Doxolen", batch no. 83520, molecular weight: 580.00 (C27H29NO11·HCl) (CAS 23214-92-8) manufactured by Eurofarma Laboratories and distributed by Zodiac Pharmaceuticals SA, Sao Paulo, Brazil, were used for the experiment. Preparation of the DXR at a concentration of 0.4 mM occurred immediately before the experiment.

2.2. Multi-walled carbon nanotubes

Multi-walled, functionalized carbon nanotubes were provided by the Institute of Chemistry, University of Campinas (UNICAMP), Campinas, São Paulo. The raw multi-walled carbon nanotubes (MWCNT) (1.0 g) supplied from CNT Co., Ltd. (Korea) were treated by reflux technique and magnetic stirring with HNO3 (6 mol L^{-1}) for 24 h at 150 °C. After cooling down to room temperature, they were vacuum-filtered through a 0.2 μm PTFE membrane and washed with deionized water until the filtrate reached a neutral pH. The treated multiwall carbon nanotubes were dried in vacuum system at room temperature for 24 h and this sample was named "NTC-3". Chemical vapor deposition (CVD) was used an oxidation temperature of 595 °C. A high quality hydrocarbon, with a metal as catalyst, was used as a source of carbon. The purity of the resulting nanotubes was 98.5%, with only 1.5% iron oxide.

The size and morphology of the MWCNT were characterized by using a transmission electron microscope (TEM/Carl Zeiss CEM-902) and scanning electron microscopy (SEM/FEI NanoLab200). Specific surface area was measured by adsorption, using the BET (Brunauer–Emmett–Teller). MWCNTs had a specific surface area of 298 g/m^2, contained diameter 10–40 nm and contained length 1–2 μm. Among the most common tools to characterize the morphologies and dimensions of as-produced MWCNTs (in powder form), scanning electron microscopy (SEM) and transmission electron microscope (TEM) is by far the most popular. At the present, SEM and TEM imaging are used to characterize the overall morphology of MWCNT samples, and could also be used to quantify the degree of purity within samples, as well as the dimensions of the tubules. Fig. 1 shows a transmission electronic microscope (TEM) and scanning electronic microscopy (SEM) image showing the nano-structure of a multi-walled carbon used in this study.

2.3. Drosophila melanogaster strains

For testing with SMART, mutant strains of *D. melanogaster* were provided by Dr. Urich Graf of the Institute of Toxicology, University of Zurich, Shwerzenbach, Switzerland. These strains included: *mwh*, *flr*^{3} and *ORR*, with *multiple wing hairs* (*mwh*, 3–0.3) and *flare-3* (*flr*^{3}, 3–38.8). They were stored in ¼ L flasks with a culture medium of 820 mL of water, 25 g yeast (*Saccharomyces cerevisiae*), 11 g of agar, 156 g of banana and 1 g of Nipagin. The medium was stored in a BOD incubator at a temperature of ±25 °C with relative humidity of 67%.

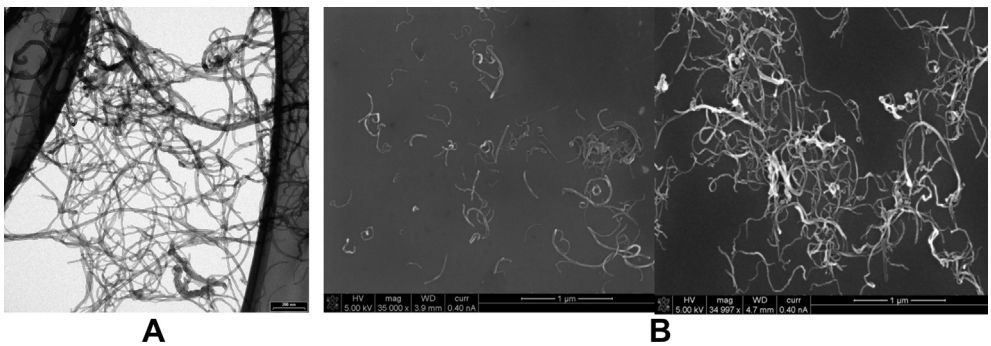

Fig. 1. Morphology of the carbon nanotubes: (A) TEM - transmission electron micrographs of dry nanopowders (measure bar is 200 nm) and (B) SEM - scanning electronic microscopy (SEM).

Two types of crosses were made for the experiment: Standard cross, ST – using *flr*^{3}/*In(3LR)TM3, ri p*^{p} *sep I*(3)*89Aa bx*^{34e} *e Bd*^{S} virgin females crossed with *mwh/mwh* (Frölich and Würgler, 1989) males; and a cross with High bioactivation (HB) virgin females *ORR/ORR; flr*^{3}/*In(3LR)TM3, ri p*^{p} *sep I(3)89Aa bx*^{34e} *e Bd*^{s} with *mwh/mwh* males (Graf et al., 1984).

These crosses produced progeny of two types: individuals trans-heterozygous for the marker genes (MH), and heterozygous for the TM3 (BH) chromosome (Graf et al., 1984; Andrade and Lehmann, 2003). The BH Individuals differ phenotypically from the MH individuals. They have clipped wings, a characteristic *Bd*^{S} marker, giving a serrated appearance (Graf et al., 1984). This study, however, analyzed only the MH individuals.

2.4. Somatic mutation and recombination test (SMART) on Drosophila melanogaster

Eggs were collected from the progeny of ST and HB crosses over a period of 8 h in flasks containing a solid agar base (3% agar in water) and a layer of yeast supplemented with sucrose. After 72 ± 4 h, the larvae were rinsed with reverse osmosis water and collected by using a fine-mesh sieve. These larvae were transferred to 25 mL flasks containing 1.5 g of mashed potatoes (HIKARI® São Paulo, Brazil) rehydrated with 5 mL of varying concentrations of carbon nanotubes (50, 100, 150, 200 and 250 mg/mL). The larvae were fed on this medium for the rest of their development (48 h). The concentrations were based on studies from Franchi et al. (2009), who used the concentrations of 50, 100, and 200 μg/mL of MWCNT to assess cell viability (XTT kit Roche). Liu et al. (2009) used concentration in food containing 0, 100 or 1000 μg-nanomaterial/g-food.

All dilutions were made in reverse osmosis water because they are functionalized carbon nanotubes. For a positive control, doxorubicin (DXR 0.4 mM) was used, and for negative control reverse osmosis water was used. The DXR was used as positive control because in *D. melanogaster*, using SMART, it was classified as a strong mutagen inducing all types of spots (Orsolin et al., 2012). Third stage larvae were subjected to a chronic treatment, during approximately 48 h, until development of the pupal stage.

After undergoing metamorphosis, the adult flies were transferred to vessels containing 70% ethanol, and mounted on slides. The wings of the flies were removed and examined under a stereoscopic microscope using a pair of entomological tweezers. They were mounted in Faure [gum arabic (30 g), glycerol (20 mL), water (50 mL) and chloral hydrate (50 g)] and analyzed in an optical microscope with magnification of 400 times (Graf et al., 1984). Analysis of the trichomes, present on the dorsal and ventral surfaces of the wings, permitted the identification of wing spots and mutant hairs that could be classified as simple (*mwh* or *flr*^{3}) or twin (*mwh* and *flr*^{3}). The analysis also recorded microscopic lesion type (single or twin), the size (small – with mutant cells 1–2 or large – 3 or more mutated cells) and the location where the spots were found.

All compounds were tested in two different experiments. The data were combined following verification that the two independent experiments produced acceptable reproducibility. The concentrations used in these experiments were based on studies of cell viability and clonogenicity performed by Franchi et al. (2009) and toxicity of carbon nanomaterials in *Drosophila* (Liu et al., 2009). To assess the cytotoxicity of the concentrations, numbers of the adult flies in the tubes treated with MWCNT were counted.

A test for cytotoxicity was performed in tubes of 100 larvae each, exposed to the concentrations of carbon nanotubes tested. The number of surviving flies per treatment provided an indicator of the toxicity of the compound.

2.5. Statistical analysis

To assess the statistical significance of the results the procedure proposed by Frei and Würgler (1988) was performed. This is a multiple decision analysis that generates four different diagnoses: positive, weak positive, negative or inconclusive. The non-parametric *U* test of Mann, Whitney and Wilcoxon tests were used to exclude false positive results (Frei and Würgler, 1995).

3. Results

The survival rate observed in all concentrations used revealed the levels of toxicity at all of the doses tested (Fig. 2). The treatments showed survival rates higher than 75% at all concentrations in the descendants from the ST and HB crosses. There is a small difference in survival rates in MH individuals of the ST cross and HB cross. The results corroborate the findings of Liu et al. (2009) who reported a lack of toxicity detected in a test of larval *D. melanogaster* treated with carbon nanotubes. According to Liu et al. (2009), in high doses (1000 μg/g), there was a delay, although not significant, in the physical development of the larva.

Tables 1 and 2 present the results obtained from the mutagenic evaluations with carbon nanotubes at concentrations of 50, 100, 150, 200 and 250 μg/mL, positive control (DXR 0.4 mM) and negative control (reverse osmosis water). DXR produced a positive response in both descendants of the ST cross and HB cross, indicating that DXR is mutagenic in the assay. The data shows that mutant spots in all its categories (small, large twin and total spots) are observed in MH individuals of the ST and HB crosses, when treated with different concentrations of MWCNT, did not increase, and were not statistically significant ($P > 0.05$), compared with the frequency of spots observed in the negative control (Tables 1 and 2). There was, therefore, no need to analyze the BH individuals.

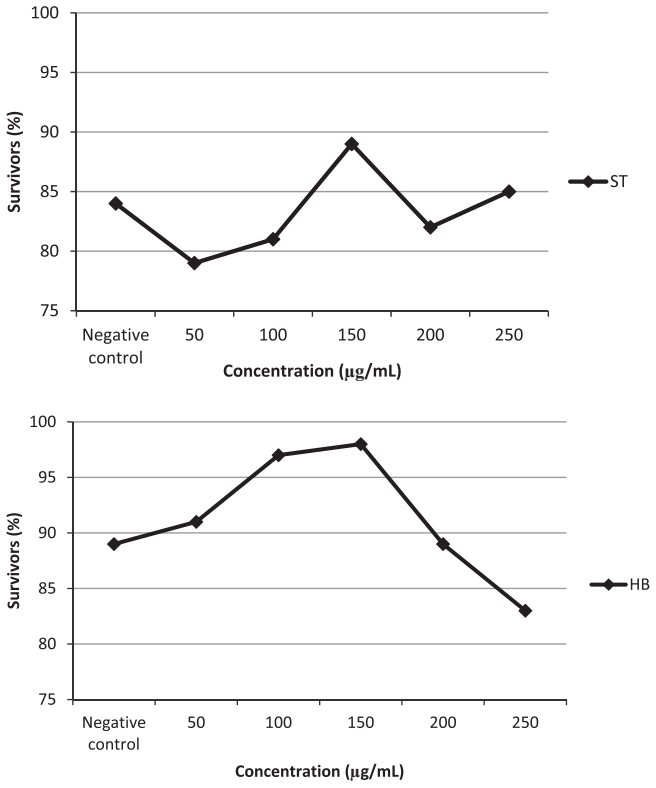

Fig. 2. Relative percentage of survival of flies detected after metamorphosis of third-stage larvae treated with different concentrations of multi-walled, functionalized carbon nanotubes (MWCNT).

The Fig. 1 shows a transmission electronic microscope (TEM) and scanning electronic microscopy (SEM) image showing the nano-structure of a multi-walled carbon used in this study.

The Fig. 3 shows *Drosophila* larvae fed with suspended carbon nanotubes. (A) Larvae not fed nanotubes (Control). (B) Carbon nanotubes in the food are taken into the larval gut (black areas compared to control). Optical micrographs indicate uniform dispersion of MWCNT in the body of larvae. Low but uniform dispersion of nanotubes in the gut of *Drosophila* can be seen.

4. Discussion

Carbon nanotubes (CNTs) are allotropes of carbon with a cylindrical nanostructure. These cylindrical carbon molecules have unusual properties, which are valuable for nanotechnology, electronics, optics and other fields of materials science and technology. The toxicity of carbon nanotubes has been an important question in nanotechnology. Such research has just begun. The data are still fragmentary and subject to criticism. The preliminary results highlight the difficulties in evaluating the toxicity of this heterogeneous material. In our study the main objective was to evaluate the mutagenicity of multi-walled, functionalized carbon nanotubes

Table 1
Frequency of mutants spots observed in the marked trans-heterozygous descendants (MH) of *Drosophila melanogaster* derived from the standard (ST) cross treated with different concentrations of carbon nanotubes (50, 100, 150, 200, 250 μg/mL), positive control (DXR 0.4 mM) and negative control (reverse osmosis water).

Genotype and Compounds	No. of files (*N*)	Spots per fly (no. of spots) statistical diagnosis[a]				Total *mwh* clones[c] (*n*)
		Small single spots (1-2 cells)[b] *m* = 2	Large single spots (>2 cells)[b] *m* = 5	Twin spots *m* = 5	Total spots *m* = 2	
mwh/flr^3						
Negative control	40	0.30 (12)	0.10 (04)	0.05 (02)	0.45 (18)	18
DXR 0.4 mM	60	0.80 (48) +	1.10 (66) +	0.83 (50) +	2.73 (164) +	154
50	60	0.33 (20) i	0.03 (02) –	0.02 (01) i	0.38 (23) –	22
100	60	0.40 (24) i	0.07 (04) i	0.00 (00) i	0.47 (28) –	28
150	60	0.50 (30) i	0.03 (02) –	0.00 (00) i	0.53 (32) i	32
200	60	0.30 (18) i	0.05 (03) i	0.02 (01) i	0.37 (22) –	22
250	60	0.43 (26) i	0.08 (05) i	0.02 (01) i	0.53 (32) i	31

For negative control reverse osmosis water was used. *m*, multiplication factor for the evaluation of results significantly negative. Significance level $\alpha = 0.05$. DXR, doxorubicin.
[a] Statistical diagnosis according to Frei and Würgler (1988): + positive; - negative; i, inconclusive.
[b] Including simple spots *flr*^{3} rare.
[c] Considering mwh clones for *mwh* single spots and twin spots.

(MWCNT) in somatic cells of *D. melanogaster*, using the somatic mutation and recombination test (SMART).

There was no statistically significant increase in the marked trans heterozygous descendants (MH) of the standard cross (ST) or in the total number of spots at any of the five MWNTs concentrations, compared with the spot frequencies observed in the negative control. For this reason, the balanced heterozygous descendants (BH) were not analyzed. The influence of differences in the level of cytochrome P450 on the genotoxic properties of MWNTs using the high bioactivation (HB) cross of *Drosophila* was also investigated. The HB cross is characterized by an increase in cytochrome P450-dependent bioactivation capacity for promutagens as compared with that of the ST.

Some studies describe the cytochrome P450 deposition onto carbon nanotubes (Rivas et al., 2002; Wang, 2005; Baj-Rossi et al., 2012). Zongfei et al. (2009) showed that a high dose of MWCNT can induce hepatic toxicity in mice by alternation of gene expression in the CYP450 pathway. Based on these considerations, the influence of differences in the level of cytochrome P450 on the mutagenic properties of carbon nanotubes using the high bioactivation (HB) cross of *Drosophila* was also investigated. The HB cross was created with the objective of enhancing the performance of the SMART test in the identification of cases of activation of promutagens dependent on activation via cytochrome P450 (Andrade and Lehmann, 2003). The cytochromes are a group of enzymes that are important in the metabolism of several phytochemicals and are able to activate pro-mutagens in mutagens (Sun et al., 2000). It is important to note that, even with high levels of cytochrome P450, no statistically significant increases were observed in the frequencies of spots on MH individuals from the HB cross. Thus, the presence of high levels of cytochrome P450 found in descendants of the HB cross is not important in inducing mutant spots in *Drosophila*, when larvae were exposed to carbon nanotubes. Our results were supported by Ema et al. (2012) that found neither type exerted mutagenicity in *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537, or in *Escherichia coli* WP2*uvr*A, in the absence or presence of metabolic activation.

Considering the negative results in the induction of mutant spots by the treatments used, it was concluded that the MWCMT were unable to induce genetic toxicity related to gene mutations, chromosomal and/or recombination events in somatic cells of *D. melanogaster*. The literature associated with this topic, however, presents a series of divergent results. Several studies conducted with specific CNTs in cell culture have reported that the CNTs do exert genotoxic effects on the culture, inducing toxic effects, reduction of cell proliferation, changes in ionic conductivity, lipid peroxidation, oxidative stress, mitochondrial dysfunction and changes in cell morphology (Shvedova et al., 2003; Shvedova et al., 2009; Reddy et al., 2010). They also suggest that there is direct interaction between CNTs and DNA, or proteins related to DNA that could lead to physical damage in genetic material (Singh et al., 2009). However, Asakura et al. (2010) suggest that MWCNTs interfere physically with biological processes during cytokinesis, but not directly interacting with DNA. Petersen et al. (2013) provide evidence that carbon nanotubes can help protect DNA molecules from oxidative damage. They investigated the impact of ultrasound on a

Table 2
Frequency of mutants spots observed in the marked trans-heterozygous descendants (MH) of *Drosophila melanogaster* derived from the high bioactivation (HB) cross treated with different concentrations of carbon nanotubes (50, 100, 150, 200, 250 μg/mL), positive control (DXR 0.4 mM) and negative control (reverse osmosis water).

Genotype and Compounds	No. of files (*N*)	Spots per fly (no. of spots) statistical diagnosis[a]				Total *mwh* clones[c] (*n*)
		Small single spots (1-2 cells)[b] *m* = 2	Large single spots (>2 cells)[b] *m* = 5	Twin spots *m* = 5	Total spots *m* = 2	
mwh/flr^3						
Negative Control	60	0.92 (55)	0.10 (06)	0.05 (03)	1.07 (64)	63
DXR 0.4 mM	60	1.10 (66) –	1.25 (75) +	0.80 (48) +	3.15 (189) +	180
50	60	0.85 (51) –	0.05 (03) –	0.03 (02) i	0.93 (56) –	55
100	60	0.85 (51) –	0.03 (02) –	0.00 (00) i	0.88 (53) –	52
150	60	1.20 (72) –	0.08 (05) i	0.02 (01) i	1.30 (78) i	78
200	60	1.03 (62) –	0.08 (05) i	0.07 (04) i	1.18 (71) –	71
250	60	0.97 (58) –	0.12 (07) i	0.02 (01) i	1.10 (76) i	66

For negative control reverse osmosis water was used. *m*, multiplication factor for the evaluation of results significantly negative. Significance level $\alpha = 0.05$. DXR, doxorubicin.
[a] Statistical diagnosis according to Frei and Würgler (1988): + positive; - negative; i, inconclusive.
[b] Including simple spots *flr*^{3} rare.
[c] Considering mwh clones for *mwh* single spots and twin spots.

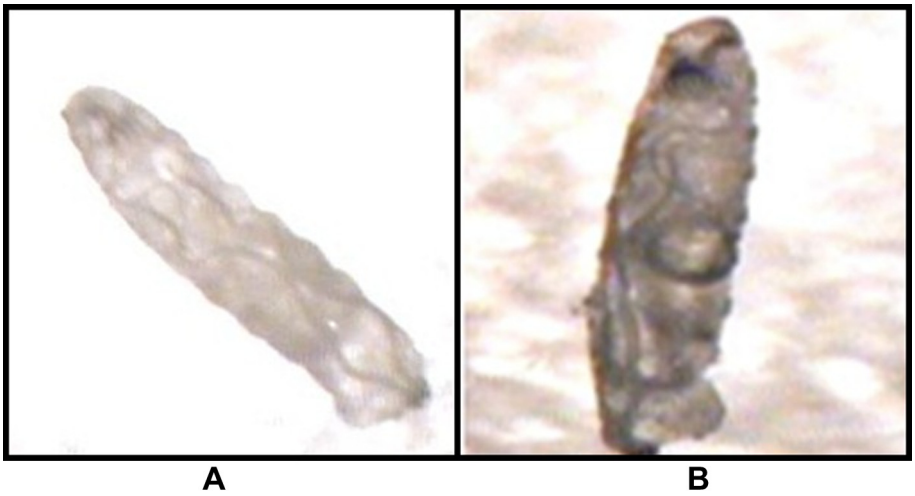

Fig. 3. *Drosophila* larvae fed with suspended carbon nanotubes. (A) Larvae not fed nanotubes (Control). (B) Carbon nanotubes in the food are taken into the larval gut (black areas compared to control).

solution of DNA fragments known as oligomers, in the presence and absence of carbon nanotubes. It was verified that the CNTs can act as scavengers, connecting the oxidative species in solution and preventing them from interacting with DNA. It should be noted that one component missing in these studies is an understanding of what actually physically happens at the molecular level.

Differently to what was observed in our studies, one factor which must be taken into account in the mutagenicity studies of CNTs are the metallic impurities emanating from the catalysts during their growth, which are present in the samples. These impurities can be observed in ends, interior walls, and other nanostructures. They can be trapped in graphitic cavities and may or may not be linked to carbon atoms. These metals can generate ROS (reactive oxygen species) in biological systems, inducing a state of oxidative stress, a major effect in the determination of the mutagenicity of samples of CNTs (Shvedova et al., 2003).

Although cell viability assays performed by a few researchers have detected that SWCNT samples containing residual iron have a high cytotoxic potential (Franchi et al., 2011), the MWCNT samples used in the present study catalyzed with C, O and Fe demonstrated no cytotoxicity according to the test applied. This finding may be associated with the high levels of purity of the nanotubes used in our study: only 1.5% from iron oxide and the other 98.5% from carbon. The purity of the nanotubes may be due to the method of manufacture and purification used in the samples. The CVD technique (chemical vapor deposition) for producing CNTs follows procedures that have greater control of the major parameters that can generate impurities. The heat treatment, which attempts to separate the synthesis products depending on their size and the chemical treatment that separates the products of the synthesis by reactivity, are both positive in the elimination of a large amount of impurities such as those generated by the catalysts (Jauris et al., 2011). Additionally, studies have demonstrated that nanotubes, when functionalized, are in a disaggregated state which results in lower toxicity, in cultured cells (Oliveira et al., 2011).

In our studies were used functionalized MWCNT. The functionalization of the CNTs results in their becoming more biocompatible, facilitating their interaction with organic molecules, biological or other chemical groups such as pharmaceuticals or DNA (Sinnott, 2002). Thus, the absence of mutagenicity verified in our study may also be associated with the use of functionalized CNTs. The functionalization of the CNT can drastically modify its properties, such as solubility, reactivity, and electronic properties. This modification is consistent with the objectives of functionalization: to make possible the application of CNTs in systems that depend directly on the neutralization of tube to tube interactions. These "Van der Waals" interactions are responsible for the high hidrofobicity of the tubes and create difficulty in their application, principally in the biological area. The neutralization of these interaction forces is thus decisive for the application of nanotubes in biological systems. For this reason the CNTs must be water soluble so that biocompatibility can be studied (Nascimento, 2008).

On the other hand, when nonfunctionalized nanoparticles accumulate in clusters, due to their hydrophobic characteristics, biological responses are controversial since CNTs solutions containing many agglomerates, exhibit negative mutagenic responses (Singh et al., 2009). Few studies have investigated the interactions of nanoparticles in *Drosophila*. But Leeuw et al. (2007), in a study of single-walled nanotubes (SWCNTs), using fluorescence microscopy, evaluated changes in concentrations of nanotubes among tissues of *Drosophila*. The videos, made from sequences of the fluorescence images, show clearly the peristalsis of the digestive system. It is observed that the nanotubes ingested pass through the digestive system and only a small fraction are incorporated in tissues. To determine whether any of SWCNTs ingested penetrate the wall of the intestine and enter the interior of the larvae, individual tissues were removed, fixed, and analyzed for NIR fluorescence. It was found that low levels of nanotube fluorescence ($\sim 10^{-8}$) were detected in all of the tissues examined.

These data suggest that the absence of the mutagenic effect, observed in our study, may also be related to the small amount of nanotubes found embedded in the tissues. These results corroborate the findings of Liu et al. (2009) who reported a lack of toxicity detected in a test of larval *D. melanogaster* treated with carbon nanotubes. According to Liu et al. (2009), in high doses (1000 μg/g), there was a delay, although not significant, in the physical development of the larva.

We verify, by means of optical microscopy (dark concentrations in tissues) one a low but uniform dispersion of nonotubes in the gut of *Drosophila*, as can be seen in Fig. 3. The amount that penetrated into the core of the imaginal disk cells was probably not sufficient to cause mutagenic effects in the organism tested. We did not analyze the accumulation of nanotubes directly in imaginal disk cells. However, Leeuw et al. (2007) found the presence of low levels of nanotubes in the cells of the imaginal disk in *Drosophila*. They believe that this accumulation most probably represents a secondary uptake after entry of SWNTs into the hemolymph. Nev-

ertheless, a variety of mechanisms may influence the mutagenic profile of these nanomaterials. The experimental strategy used in our study highlighted the absence of mutagenic effects of functionalized MWCNTs in the somatic cells of *D. melanogaster*. It is possible; however, that several mechanisms influenced the results of the toxicity tests of the biomaterial. For example, the structure, the extent of clustering and the purity of CNTs together with the functionalization that was applied could have modified characteristics such as solubility, reactivity and electronic properties. For these reasons, further investigations *in vivo* must be performed before any clinical application and/or industrial use of CNTs can be confirmed.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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