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Neurobehavioral toxicity of carbon nanotubes in mice: Focus on brain-derived neurotrophic factor mRNA and protein

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Abstract

Objectives: The aim of this study was to evaluate neurobehavioral toxicity of single-walled (SWNTs) and multiwalled carbon nanotubes (MWNTs) in mice. **Methods:** Male NMRI mice were randomized into 5 groups ($n = 10$ each): Normal control (NC) group was injected intraperitoneally (i.p.) with phosphate-buffered saline (PBS) solution (pH 7.8; ca. 1 mL), MW80 and MW800 groups were injected with either i.p. 80 or 800 mg kg^{-1} MWNTs suspended in 1 mL of PBS and SW80 and SW800 groups were injected with either i.p. 80 or 800 mg kg^{-1} SWNTs suspended in 1 mL of PBS. After 2 weeks, five mice from each group were evaluated for brain-derived neurotrophic factor (BDNF) messenger RNA expression and protein content of brain tissues. Locomotion, anxiety, learning and memory, and depression were measured by open field test (OFT), elevated plus-maze (EPM), object recognition test (ORT), and forced swimming test (FST), respectively. **Results:** Ambulation time and center arena time in the OFT did not change among groups. In the EPM paradigm, SWNTs (800 mg kg^{-1}) and MWNTs (80 and 800 mg kg^{-1}) showed an anxiogenic effect. In ORT, MWNTs (80 mg kg^{-1}) increased the discrimination ratio while in FST, MWNTs showed a depressant effect as compared to vehicle. The BDNF gene expression in mice treated with 80 and 800 mg kg^{-1} SWNTs or 80 mg kg^{-1} MWNTs decreased as compared to NC mice although BDNF gene expression increased in mice that were treated with 800 mg kg^{-1} MWNTs. The whole brain BDNF protein content did not change among groups. **Conclusion:** Our study showed that i.p. exposure to carbon nanotubes (CNTs) may result in behavioral toxicity linked with expression of depression or anxiety that depends on the type of CNTs. In addition, exposure to CNTs changed BDNF gene expression.

Keywords

Carbon nanotubes, brain-derived neurotrophic factor, memory, anxiety, depression, locomotor

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Introduction

We have witnessed application of carbon nanotubes (CNTs) in both industry and biomedicine due to their unique intrinsic physical and chemical characteristics (Fabbro et al., 2011). The CNTs are seamless cylindrical nanostructures of graphene sheets that roll up to form hollow tubes closed at each end by hemispherical fullerene caps. These fiber-shaped allotropes of carbon mainly present as single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs).

A large body of literature has reported potential (eco)toxicities of CNTs in both the environment and humans (see review Ema et al., 2015; Jones and Grainger, 2009; Lacerda et al., 2006). According to these seminal reviews, the physicochemical characteristics of CNTs (length, diameter, surface area, tendency to agglomerate, bio-durability, presence and nature of catalyst residues, as well as chemical functionalization) are key determinants of their interactions with living organisms and their toxicities. Beside the physicochemical properties of CNTs, how they enter the body of mammals, especially humans, influence their toxicities (Bihari et al., 2010; Yang et al., 2013).

In this continuum, many reports deal with possible (neuro)toxicities of CNTs with the aid of several *in vitro* and *in vivo* batteries (e.g. Chen and Hsiue, 2013; Ema et al., 2012) but their mild neurotoxicities are still not definitely determined. Since the neurobehavioral toxicity of intraperitoneal (i.p.) exposure to CNTs had not been evaluated, no evidence was available to enable prediction of behavioral functions that would most likely be affected by these putative nanotoxicants. Furthermore, there is need for a molecular biomarker that could be used as an endpoint to evaluate neurobehavioral toxicities. A growing number of clinical and experimental evidence reports that brain-derived neurotrophic factor (BDNF) and BDNF/TrkB signaling plays a pivotal role in neuronal survival, axon growth, neuronal transmission, synaptic plasticity, and learning and memory formation (Lewin and Barde, 1996; Minichiello, 2009; Yoshii and Constantine-Paton, 2010; Zhang et al., 2012). Therefore, we examined the effects of pristine CNTs on the behavior of male mice, paying specific attention to any changes in BDNF.

Materials and methods

Particle characteristics

The CNTs were synthesized at Research Institute of Petroleum Industry (Tehran, Iran) using a catalytic chemical vapor deposition method (Kim et al., 2005; Figure 1). Raman spectroscopy showed that synthesized SWNTs and MWNTs consisted of 99% and 95% carbon and small amounts of cobalt and iron catalysts, respectively (Figures 2 and 3). The nominal characteristics of the CNTs were as follows: thermal conductivity (w mv^{-1}), 3000 (SWNTs) and 1500 (MWNTs); specific surface area (m^2 g^{-1}), 700 (SWNTs) and 270 (MWNTs); outside diameter ranges (nm), 1–2 (SWNTs) and 10–30 (MWNTs); internal diameter ranges (nm), 0.8–1.1 (SWNTs); and average fiber length (μm), 10 for both SWNTs and MWNTs. Heat-treated particles CNTs were suspended in sterile phosphate-buffered saline (PBS) solution and sonicated at 70 W and 42 Hz for 10 h/day for 5 days to prepare a suspension. The resulting suspension was resonicated using dynamic light scattering for 0.5 h just before administration.

Animal subjects

Male adult Naval Medical Research Institute (NMRI) mice (*Mus musculus* L.) of 25 g average weight were housed in standard metal cages maintained at 22 $\pm$ 2°C, 40–60% RH under a 12-h light:12h dark photocycle. Mice were administered pelleted feed (Gharbdaneh Co., Kermanshah, Iran) and tap water *ad libitum* except when animals were feed- or water-restricted during psychobiological tests.

Fifty mice were randomized into 5 groups ($n = 10$ for each group): Normal control (NC) group was i.p. injected with PBS solution (pH 7.8; ca. 1 mL) as vehicle, two MWNT-treated groups, MW80 and MW800, were i.p. injected with 80 and 800 mg kg^{-1} MWNTs suspended in 1 mL of PBS and two SWNT-treated groups, SW80 and SW800, were i.p. injected with 80 and 800 mg kg^{-1} of SWNTs suspended in 1 mL of PBS. After two weeks, half the number of each group were used for the evaluation of the biochemical and/or psychobiological endpoints (*vide infra*). All experiments were approved by the Medical Ethical Committee of our institute.

Psychobiological endpoints

Open field test. Locomotor and exploratory activities of animals were evaluated by open field test (OFT). Mice were placed for 5 min in an open field apparatus that consisted of a bright square measuring 100 cm across. The time of movement including walking or running (ambulation) and the time spent in the central

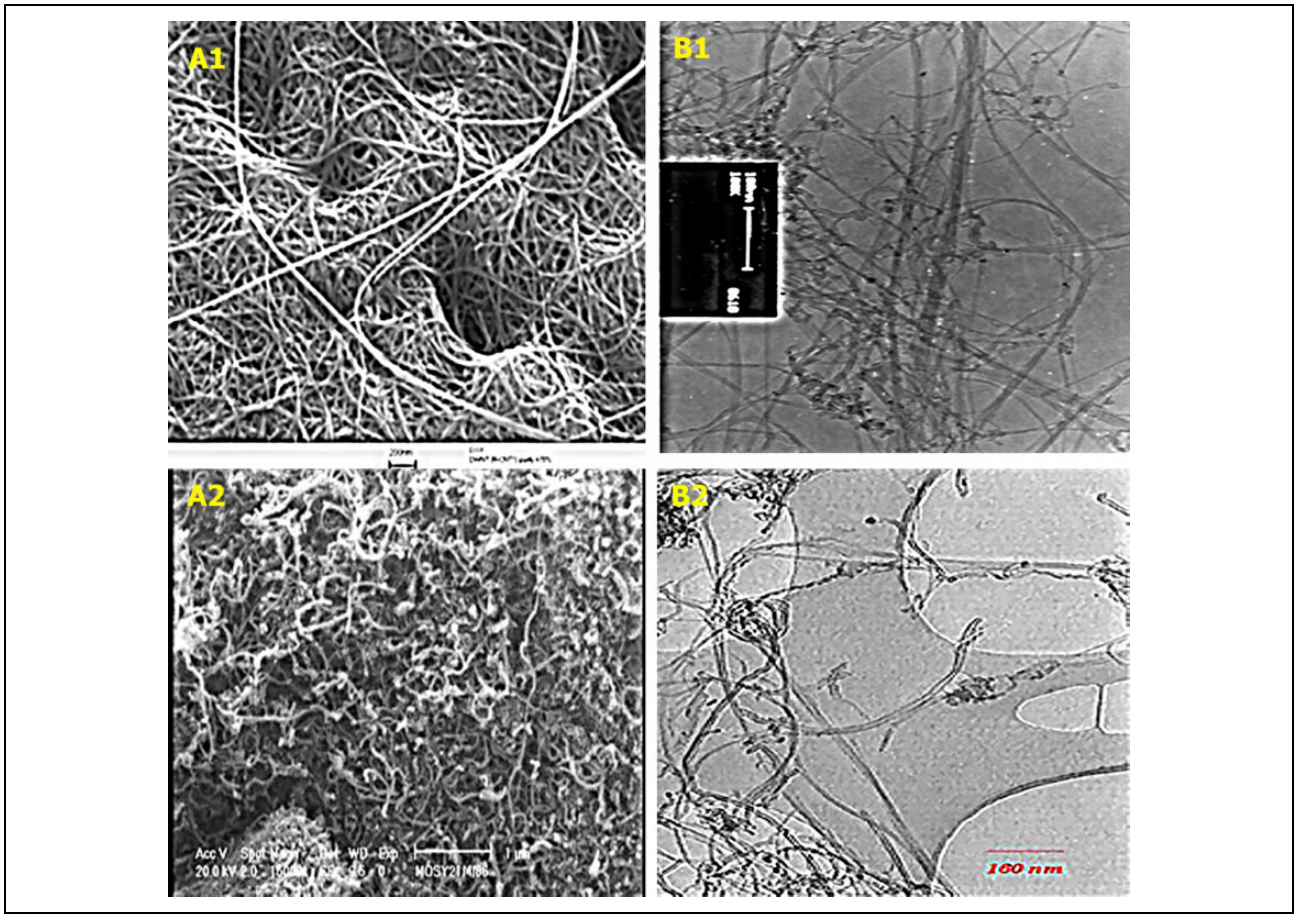

Figure 1. Scanning electron microscope (A1 and A2) and transmission electron microscope (B1 and B2) photographs of single- and multiwalled carbon nanotubes, respectively. Courtesy of Research Institute of Petroleum Industry, Tehran, Iran.

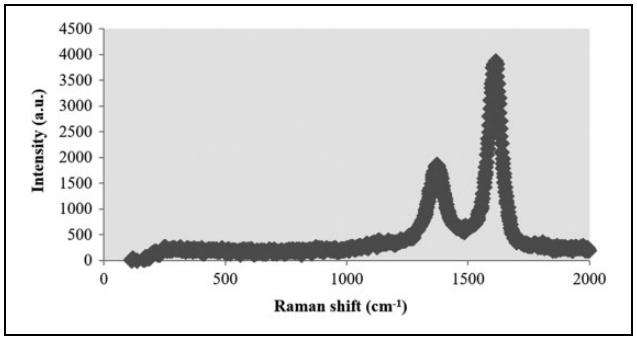

Figure 2. Raman spectrum of multiwalled carbon nanotubes.

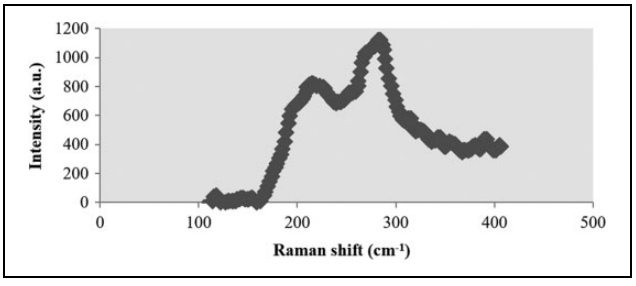

Figure 3. Raman spectrum of single-walled carbon nanotubes.

parts of the arena were recorded by a camera at a right angle above the open field apparatus as locomotor activity and anxiety marker, respectively (Ivani et al., 2012).

Elevated plus-maze. The anxiety-associated behavior of mice was explored after CNTs intake using an elevated plus maze (EPM). The wooden apparatus comprised four arms (50 cm long × 10 cm wide) configured in a plus manner situated 50 cm high, with two opposing arms constructed with 50-cm-tall walls. Mice were placed in the center square and their behavior was video-taped for 5 min and their videos were evaluated for following parameters: (1) time spent in the open arms, (2) time spent in the closed arms,

(3) number of entries into the open arms, and (4) number of entries into the closed arms. Open arm activity was the time that mice spent in the open arms relative to the total amount of time spent in any arm (open/total × 100), and the number of entries into the open arms was quantified relative to the total number of entries into any arm (open/total × 100). The total arm entries were considered as locomotor activity to ensure that there are no differences in overall activity levels in studied groups (Degroot et al., 2001).

Object recognition test. Object recognition test (ORT) was used to assess the effect of CNTs in learning and memory (Miraghaee et al., 2011). In short, mice were habituated in an empty arena for 5 min. Twenty four hours after habituation, two identical objects were presented in the open field, and mice were allowed to explore them freely for 20 min for 5 successive days. On day 6, the mice were allowed to explore the open field in the presence of the familiar object and a novel object in 3 min, and times spent exploring each object were recorded to test long-term recognition memory. Exploring was defined as an investigation within 0.7 cm around the object and/or touching it with the nose. In this way, the following variables were defined: $A =$ the time spent exploring old object, $B =$ the time spent exploring novel object, and $B - A/B + A$ was considered as the discrimination ratio.

Forced swimming test. The forced swimming test (FST) was used to evaluate behavioral despair or depression in mice after CNTs intake as described previously (Miraghaee et al., 2011). Briefly, mice were forced to swim individually in a glass jar (20 cm diameter, 46 cm height, and 30 cm water at a temperature of 25 ± 3°C) for a period of 6 min in a test session and the following video-taped behaviors were rated 2 min after beginning the test that lasted for 5 min: (1) immobility—time a mouse remains floating without vigorous struggling; (2) swimming—time a mouse shows active motions around in the jar.

Real-time (RT)-qPCR assay. The mice were killed under deep anesthesia with i.p. ketamine (80 mg kg^{-1}; Alfasan Co, the Netherland)/diazepam (0.5 mg kg^{-1}; Chemi Darou Co., Iran) cocktail injection 2 weeks after i.p. exposure. Subsequently the whole brain was removed, frozen in liquid nitrogen, and stored at −80°C until analysis. Total RNA in brain tissues was isolated using a commercial kit (Thermoscientific, England) as described by the manufacturer. Purity and RNA concentration were spectrophotometrically evaluated by optical density measurements at 260 and 280 nm. RNA (1 μg) was reverse transcribed using a complementary DNA (Cdna) synthesis kit (Bioneer, Germany) as described by the manufacturer.

The forward and reverse primers (Takapozist Co. Tehran, Iran) used for total mouse BDNF mRNA were 5′-TAAATGAAGTTTATACAGTACAGT GGTTCTACA and 5′-AGTTGTGCGCAAATGA CTGTTT, respectively. The forward and reverse primers used for β-actin mRNA used to normalize results were 5′-AGATTACTGCTCTGGCTCCT and 5′-CATCTGCTGGAAGGTGGACA, respectively. The primer preparation was done as described by the manufacturer. Diethylpyrocarbonate water volume was added into the primer tube in order to supply 100 pmol μL^{-1} after 30 min incubation at RT and mild pipetting; then a primer concentration of 10 pmol μL^{-1} was poured in RNase-free microtubes. SYBR-Green Master Mix solution (Ampliqon, Denmark) was prepared for real-time PCR reaction. For each sample, real-time quantitative reverse transcription (RT-qPCR) was done in microtubes including 10 μL forward primer, 10 μL reverse primer, 20 μL SYBR Green Master Mix, 5 μL cDNA with master cycler real-time PCR (Eppendorf, Germany) with the following cycling parameters: 95.0°C for 2 min (initial temperature) and 40 cycles at 95.0°C for 15 s, 56.5°C for 15 s, and 72.0°C for 15 s. The end stage was the application of a default temperature program for melting curve to make sure of the absence of primer dimers.

For relative quantification (RQ) of gene expression, the mean CT values of the triplicates of the BDNF and β-actin genes were calculated, followed by subtraction of the mean CT values of the β-actin gene to the mean CT values of the BDNF gene (delta CT values). Then, the power of all delta CT values was calculated based on the formula power $= 2^{-\text{deltaCT}}$ (Livak and Schmittgen, 2001). Finally, RQ values, presented as fold change relative to the control group, were obtained by the division of the power value of each sample by the mean power value of the control group.

ELISA assay. Whole brain tissue was homogenized in a lysis buffer containing 137 mM sodium chloride, 20 mM Tris-hydrochloride pH 8.0, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg mL^{-1} aprotinin, 0.1 mM benzethonium chloride, and 0.5 mM sodium vanadate. The homogenates

Table 1. Open field behavior after intraperitoneal treatment with SWNTs and MWNTs in mice.[a]

		SWNTs		MWNTs	
	NC	SW80	SW800	MW80	MW800
Ambulation time (s)	202.8 ± 5.0	188.8 ± 15.6	162.2 ± 15.9	199.0 ± 21.0	194.6 ± 22.4
Center arena time (s)	45.6 ± 6.2	25.0 ± 5.1	37.8 ± 8.2	38.8 ± 5.9	28.2 ± 6.0

SWNT: single-walled carbon nanotube; MWNT: multi-walled carbon nanotube; NC: normal control.
[a]Data shown as mean ± SEM: NC: normal control mice; SW80 and SW800 received 80 and 800 mg kg^{-1} SWNTs, respectively. MW80 and MW800 received 80 and 800 mg kg^{-1} MWNTs, respectively.

were then centrifuged at 3500 × *g* for 10 min, the supernatants were collected and total protein concentration was determined (Bradford, 1976) using bovine serum albumin as a standard. BDNF protein was quantified using a mouse enzyme-linked immunosorbent assay (ELISA) kit (Chongqing Biospes Co., Ltd, China) with sensitivity <2 pg mL^{-1} according to the manufacture's protocol.

Statistical analyses

The data were expressed as means and standard error of the mean. The Shapiro–Wilk test was used to determine the Gaussian distribution and Levene's test for the homogeneity of variances of the data. Nonparametric Kruskal–Wallis analysis of variance (ANOVA) was utilized when data were not normally distributed and the post hoc pair-wise Mann–Whitney test was used where the overall Kruskal–Wallis revealed a significant effect. For the normally distributed data, if ANOVA revealed a significant difference, a post hoc Tukey's test was used to check the difference between groups. Pearson's correlation test was used for the correlation analysis. Significance level was set at $p < 0.05$ and all statistical analyses were carried out using SPSS version 16.0 for Windows (SPSS, Chicago, Illinois, USA).

Results

Open field test

In OFT, ambulation time ($F(4,20) = 0.884, p = 0.491$) and center arena time ($F(4,20) = 1.709, p = 0.187$); the OFT did not yield significant group differences 24–29 days after CNT treatment (Table 1).

Elevated plus maze

In the EPM paradigm, there were significant differences in the time spent on the open arms ($F(4,22) = 7.007$, $p = 0.001$) and the time spent on the enclosed arms ($F(4,22) = 5.430$, $p = 0.003$) among groups. It can be seen from Table 2 that doses of SWNTs (800 mg/kg) and doses of MWNTs (80 and 800 mg kg^{-1}) significantly decreased the time spent on the open arms in mice compared to control animals that were tested 19 days after injection, indicating an anxiogenic effect. In this continuum, only a high dose of SWNTs provoked an anxiogenic effect while the lower dose was ineffective (Table 2). The time spent in the enclosed arms significantly increased in animals treated with 80 mg kg^{-1} of MWNTs compared with control groups ($p = 0.001$; Table 2). The percentages of time spent in both open and enclosed arms were significantly different among studied groups ($F(4,22) = 6.593$, $p = 0.001$). In this regard, SWNT- and MWNT-treated groups did not show differences in both percentages of time spent on both open and enclosed arms ($p > 0.05$; Table 2). However, MWNT-treated groups showed a significant decrease in the percentage of time spent in the open arms in comparison to the normal group ($p < 0.05$; Table 2). In addition, SWNTs (800 mg kg^{-1}) and MWNTs (80 and 800 mg kg^{-1}) showed a significant increase in the percentage of time spent in the closed arm compared to the control group ($p < 0.05$; Table 2).

There were no significant changes in locomotor activity measured as the number of open ($F(4,22) = 0.300$, $p = 0.875$) and enclosed ($F(4,22) = 1.236$, $p = 0.324$) arm entries among the studied groups were similar (Table 2). The percentages of open and enclosed arm entries ($F(4,22) = 0.852$, $p = 0.508$) after both doses of SWNTs and MWNTs were also similar among studied groups (Table 2). In this study, the total number of entries into any arm was significantly different among groups ($F(4,22) = 4.128$, $p = 0.012$). The post hoc Tukey's test indicated that SWNTs-treated groups and MWNTs group that was treated with 800 mg kg^{-1} showed significant

Table 2. Plus-maze behavior after intraperitoneal treatment with SWNTs and MWNTs in mice.[a]

		SWNTs		MWNTs	
	NC	SW80	SW800	MW80	MW800
Open arm time (s)	96.7 ± 10.4	70.2 ± 12.2[b,c]	59.2 ± 0.8[c]	38.2 ± 1.1[c]	56.6 ± 7.7[c]
Enclosed arm time (s)	165.2 ± 9.9[b]	198.2 ± 13.4[b,c]	209.6 ± 10.9[b,c]	239.0 ± 6.5[c]	202.6 ± 16.9[b,c]
Open arm activity (%)	36.8 ± 3.8[b]	26.2 ± 4.7[b,c]	22.1 ± 0.7[b,c]	13.8 ± 0.4[c]	22.3 ± 4.0[c]
Enclosed arm activity (%)	63.1 ± 3.8[b]	73.7 ± 4.7[b,c]	77.8 ± 0.7[c]	86.1 ± 0.4[c]	77.6 ± 4.0[c]
Open arm entry (no.)	5.8 ± 0.6	5.8 ± 0.7	5.2 ± 0.7	4.8 ± 0.9	5.6 ± 0.9
Enclosed arm entry (no.)	10.8 ± 1.5	12.2 ± 1.2	12.4 ± 1.0	8.6 ± 1.5	11.4 ± 0.8
Percent open arm entry	29.9 ± 2.6	32.2 ± 2.9	29.2 ± 2.4	35.9 ± 3.0	32.0 ± 2.7
Percent enclosed arm entry	70.0 ± 2.6	67.7 ± 2.9	70.8 ± 2.4	64.0 ± 3.0	67.9 ± 2.7
Total arm entry (no.)	5.8 ± 3.7[b]	18.0 ± 1.5[b,c]	17.6 ± 1.6[c]	13.4 ± 2.3[b,c]	17.0 ± 1.6[c,c]

SWNT: single-walled carbon nanotube; MWNT: multi-walled carbon nanotube; NC: normal control at $p < 0.05$.
[a]Data shown as mean ± SEM: in rows, values with different letters are significantly different. NC: normal control mice; SW80 and SW800 received 80 and 800 mg kg^{-1} SWNTs, respectively. MW80 and MW800 received 80 and 800 mg kg^{-1} MWNTs, respectively.

Table 3. Object recognition test after intraperitoneal treatment with single-walled (SWNTs) and multiwalled (MWNTs) carbon nanotubes in mice.[a]

		SWNTs		MWNTs	
	NC	SW80	SW800	MW80	MW800
Exploration of old object	5.2 ± 0.6	3.8 ± 0.3	4.8 ± 0.5	3.8 ± 0.6	3.6 ± 0.6
Exploration of novel object	9.2 ± 0.6	8.6 ± 1.3	12.6 ± 1.4	11.4 ± 1.6	10.6 ± 1.7
Discrimination ratio	0.28 ± 0.04[b]	0.35 ± 0.08[b,c]	0.44 ± 0.01[b,c]	0.50 ± 0.02[c]	0.48 ± 0.04[b,c]

SWNT: single-walled carbon nanotube; MWNT: multi-walled carbon nanotube; NC: normal control at $p < 0.05$.
[a]Data shown as mean ± SEM: in rows, values with different letters are significantly different. NC: normal control mice; SW80 and SW800 received 80 and 800 mg kg^{-1} SWNTs, respectively. MW80 and MW800 received 80 and 800 mg kg^{-1} MWNTs, respectively. A discrimination ratio was the difference between time spent interacting with the novel object and the familiar object divided by the total interaction time with two objects.

differences in the total number of entries as compared to the control group ($p < 0.05$; Table 2).

Object recognition test

There were no significant differences in the total time spent exploring familiar ($F(4,20) = 1.477$, $p = 0.247$) and novel ($F(4,20) = 1.293$, $p = 0.306$) objects between control and CNT-treated groups 20–22 days after CNTs exposure (Table 3). However, the discrimination ratio showed significant differences among studied groups ($F(4,20) = 3.246$, $p = 0.033$). The post hoc Tukey's test indicated that the group treated with 80 mg kg^{-1} MWNTs showed an increase in discrimination ratio as compared to the control group ($p < 0.05$; Table 3).

Forced swimming test

The effects of CNTs on FST are shown in Table 4. ANOVA revealed a significant effect on swimming and floating (immobility) scores in the test 15–18 days after CNTs exposure ($F(4,20) = 4.932$; $p = 0.006$). Post hoc analysis revealed that MWNTs reduced swimming time and increased immobility scores in comparison to control animals ($p < 0.05$; Table 4).

Real-time PCR for BDNF mRNA expression in whole brain tissue

Whole brain mRNA levels for the housekeeping gene β-actin did not differ among studied groups and thus were used to normalize levels of BDNF mRNA. ANOVA revealed a significant effect on BDNF mRNA in studied groups ($F(3,7) = 4.971$; $p = 0.037$). Post hoc analysis revealed that BDNF mRNA did not change in the SW80 group (0.292 ± 0.254) compared to the SW800 group (0.271 ± 0.119; $p = 1.000$) and MW80 group (0.580 ± 0.167; $p = 0.936$; Figure 4). In addition, BDNF mRNA content was significantly reduced in SW80 ($p = 0.044$) and SW800 ($p = 0.042$) groups when compared to the

Table 4. Forced swimming test after intraperitoneal treatment with single-walled (SWNTs) and multiwalled (MWNTs) carbon nanotubes in mice.[a]

		SWNTs*		MWNTs*	
	NC	SW80	SW800	MW80	MW800
Swimming time (s)	101.6 ± 10.8[b]	79.8 ± 14.0[b,c]	89.6 ± 17.6[b,c]	41.4 ± 5.9[c]	46.6 ± 7.5[c]
Floating time (s)	78.4 ± 10.8[b]	100.2 ± 14.0[b,c]	90.4 ± 17.6[b,c]	138.6 ± 5.97[c]	133.4 ± 7.5[c]

SWNT: single-walled carbon nanotube; MWNT: multi-walled carbon nanotube; NC: normal control at $p < 0.05$.
[a]Data shown as mean ± SEM: in rows, values with different letters are significantly different. NC: normal control mice; SW80 and SW800 received 80 and 800 mg kg^{-1} SWNTs, respectively. MW80 and MW800 received 80 and 800 mg kg^{-1} MWNTs, respectively.

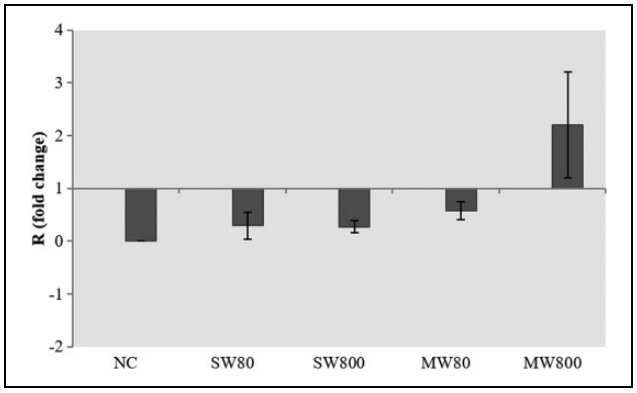

Figure 4. The effects of intraperitoneal injection of CNTs on BDNF gene expression in whole brain tissue in mice. The *y*-axis depicts the average BDNF gene expression fold change (R) between control and CNTs-treated animals; a value of 1 corresponds to the same average expression in both control and CNTs-treated animals. Bars represent mean ± SEM. NC: normal control mice; SW80 and SW800 received 80 and 800 mg kg^{-1} single-walled CNTs, respectively. MW80 and MW800 received 80 and 800 mg kg^{-1} multiwalled CNTs, respectively. CNT: carbon nanotube; BDNF: brain-derived neurotrophic factor.

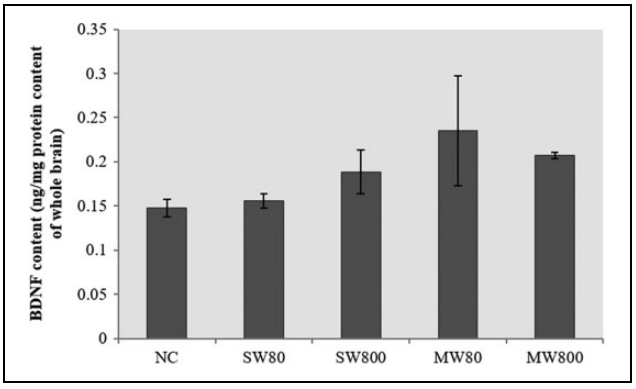

Figure 5. The effects of intraperitoneal injection of CNTs on BDNF gene expression in whole brain tissue in mice. Bars represent mean ± SEM. NC: normal control mice; SW80 and SW800 received 80 and 800 mg kg^{-1} single-walled CNTs, respectively. MW80 and MW800 received 80 and 800 mg kg^{-1} multiwalled CNTs, respectively. CNT: carbon nanotube; BDNF: brain-derived neurotrophic factor.

MW800 (2.2 ± 0.999; Figure 4) group. BDNF mRNA tended to be decreased in the SW800 group in comparison to MW80 group ($p = 0.923$; Figure 4). Two MWNTs-treated groups did not show significant differences ($p = 0.086$; Figure 4).

ELISA assay of BDNF concentration in whole brain tissue

In this continuum, CNT-treated groups did not show significant differences in whole brain BDNF protein content compared to NC mice ($F(4,11) = 1.476$; $p = 0.232$; Figure 5). The concentration of BDNF (nanograms per milligram protein content of whole brain tissue) tended to be increased in SW80 (0.156 ± 0.008), SW800 (0.188 ± 0.024), MW80 (0.235 ± 0.062), and MW800 (0.207 ± 0.003) groups in comparison to NC (0.148 ± 0.009; Figure 5). The results also showed that BDNF protein content and BDNF gene expression were not correlated ($r = 0.194$; $p = 0.567$). In addition, behavioral parameters were not correlated with BDNF protein content and BDNF gene expression ($p > 0.05$).

Discussion

In the present study, different behavioral expressions of locomotor, memory, anxiety, and depression were related to the structure of employed CNTs. In addition, relative gene expression of BDNF was different in some groups while brain BDNF protein content did not alter after i.p. exposure to CNTs. In this sense, CNTs have a great potential to enter brain and to induce oxidative stress that culminate to inflammation and behavioral disorders (Yang et al., 2010). It seems that behavioral and neurochemical modifications that occurred following i.p. exposure to CNTs

did not directly affect the nervous system *per se* since it is highly unlikely that our pristine CNTs, which have a length dimension on the order of micrometers, were part of the nanoparticles that successfully translocate to the brain through blood brain barrier (Wang et al., 2004). Interestingly, our findings indicated that high aspect-ratio MWNTs were not completely internalized by membrane structures because the fibers were longer than the membrane structure could accommodate. Bioaccumulation of pristine and functionalized CNTs in the brain tissue is scarce (e.g. Wang et al., 2004). Therefore, the behavioral modifications in the presence of i.p. exposure to CNTs would be secondary to a systemic change such as oxidative stress and inflammation of peripheral organs. Poland et al. (2008) identified inflammation and granulomatous lesions in the diaphragm of mice following i.p. injection of asbestos or CNTs, which were induced by long fibers more than short fibers. This inflammation is initiated by CNT stimulation of pro-inflammatory cytokines interleukin (IL)-1β and IL-6 and increased protein content of peritoneal lavages in mice (Qu et al., 2012). Specifically, it is the long-fiber-like properties of CNTs that cause asbestoid effects in terms of arousing inflammation (Qu et al., 2012; Yamashita et al., 2010).

Cognitive spatial memory deficits and decreased locomotor activity were found along with histopathological alterations, increased levels of oxidative stress, inflammation, and apoptosis in the brains of mice after intravenous injection of 6.25 and 12.50 mg^{-1} kg^{-1} day^{-1} SWNTs suspension (Liu et al., 2014). In the present study, both locomotor time and center arena time in OFT tended to decline in CNTs-treated groups compared with normal mice; however, these results were not statistically significant.

Jacewicz et al. (2009) showed that systemic inflammation that was induced by i.p. injection of lipopolysaccharide impairs the glutathione redox state and object recognition in male mice. Although we did not track inflammatory biomarkers in the present investigation, we cannot rule out possible inflammation (for a review, see Boyles et al., 2014). In our study, mice treated with CNTs presented similar performance to control animals in recognizing the novel and familiar objects. However, CNTs tended to increase recognition memory as measured with discrimination ratio when evaluated 1 day after training that corresponds to long-term memory. In this regard, MWNTs significantly improved recognition memory in mice as compared to that of vehicle. These results are consistent with our previous study in which mice pups born from MWNT-treated mothers had identical recognition memory in ORT as compared with control pups (Ivani et al., 2012). Based on our findings, SWNTs (800 mg kg^{-1}) and MWNTs (80 and 800 mg kg^{-1}) significantly decreased the time spent in the open arms in the EPM paradigm indicating an anxiogenic effect. In this context, the time spent in the enclosed arms increased only in animals treated with 80 mg kg^{-1} of MWNTs compared with control groups that strongly suggests an anxiogenic effect of MWNTs. In addition, all behaviors studied using EPM did not depend on the type of CNTs. In this regard, mouse pups born to mothers exposed to pristine MWNTs i.p. were identical to MWNTs used in the present study and were more anxious compared to control pups in both OFT and EPM paradigms (Ivani et al., 2012). We recently showed that mouse offspring of mothers that were i.p. exposed to 10 mg kg^{-1} pristine SWNTs identical to SWNTs used in the present study were overtly anxious but showed similar locomotor activity compared to control pups in EPM (Ivani et al., 2014). To the best of our knowledge, the molecular mechanisms that govern anxiogenic effects of CNTs have not been reported. In the present study, i.p. injection of CNTs did not alter locomotor activity as indicated by the number or percentages of enclosed arm entries. Surprisingly, both SWNTs and MWNTs at high dose (800 mg kg^{-1}) increased the total number of arm entries. Since the number of closed arm entries changes independently as percentages of the number of open arm entries and time on open arms being more accurate measures of locomotor activity (Hogg, 1996), we concluded that locomotor activity did not change following CNTs intake in EPM. In contrast to our findings, Liu et al. (2014) concluded that intravenous exposure to SWNTs (6.25 and 12.50 mg^{-1} kg^{-1} day^{-1}) can induce cognitive deficits, decreased locomotor activity, and increased oxidative stress in mice.

In the present investigation, i.p. exposure to MWNTs led to overt depressive behavior in mice while SWNTs tended to be mild depressants. Mouse pups born to mothers that were i.p. exposed to pristine MWNTs identical to MWNTs used in the present study showed significantly depressive effects. Depressive and anxiogenic potentials of CNTs may attribute to alteration of brain *milieu* particularly its oxidative status. We also measured an array of biomarkers of oxidative stress in selected tissues of mice used in this study (data not shown). In short,

pristine CNTs used in this study showed oxidative stress in liver and brain tissues after i.p. exposure. Several lines of evidence showed the putative anxiogenic role of oxidative stress (for a review, see Bouayed et al., 2009). The overt depressive effects of MWNTs in mice in the present study may be related to ongoing oxidative stress, inflammation, and cytotoxicity. Transient oxidative stress and inflammation in the plasma and liver cells along with a severe depletion of gluthathione in plasma were induced after a single dose (270 mg L^{-1}) of MWNTs (exterior diameter 15–25 nm, interior diamester 10–15 nm, surface 88 m^2 g^{-1}) functionalized with single-strand DNA (ss-DNA) administered i.p. to Wistar rats (Clichici et al., 2012). These investigators believed that coating of the nanotubes by ss-DNA did not significantly modify the surface area of the nanotubes (Clichici et al., 2012). One can expect that MWNTs functionalized with ss-DNA may behave like pristine MWNTs used in our study since the exterior diameter of our MWNTs (10–30 nm) was approximately close to Clichici et al.'s (2012) used MWNTs; however, surface area of our used MWNTs (270 m^2 g^{-1}) was considerably larger. It is obvious that the physiochemical characteristics of the nanomaterials will influence their biological behavior. In this context, long, thick, and wide MWNTs can induce inflammatory responses (Wang et al., 2009; Yamashita et al., 2010), and our results are consistent with these findings. The trace impurities of catalysts that remained inside the MWNTs, as shown by transmission electron microscopy, may not exert biological effects because the encapsulated metals are not bioavailable for at least 2 months (Clichici et al., 2012; Liu et al., 2008a, 2008b).

Both 'whole animal' and *in vitro* cell culture approaches introduce BDNF as an indicator of chemical neurotoxicity (Andersson et al., 1997; Woehrling et al., 2013). BDNF is distributed ubiquitously throughout the brain and has many roles in brain from fundamental functions such as neuronal growth, plasticity, and maintenance (e.g. Allen and Dawbarn, 2006) to elaborated cognitive functions such as long-term potentiation, learning, memory, and mood (Alonso et al., 2002; Radecki et al., 2005). Therefore we measured BDNF protein and gene expression in whole brain of CNTs-treated mice to partially characterize the mechanisms underlying possible behavioral toxicity of CNTs at the molecular level, although all behavioral endpoints were not correlated with BDNF protein and gene expression. In the present study, BDNF protein levels did not differ in whole brain tissues of CNTs-treated mice when compared to NC mice. However, BDNF protein content tended to increase following i.p. exposure to CNTs, specially MWNTs. An *in vitro* investigation showed that CNTs did not change BDNF expression levels in primary cultured neurons on CNTs-coated coverslips but stimulated BDNF release into the media (Kim et al., 2008). Nowadays, CNTs are used as artificial scaffolds to support neurite outgrowth and synapse formation, but exact mechanisms underlying interactions of CNTs and neurotrophic factors as therapeutics in disorders of central nervous system are not well understood. We also demonstrated decreased BDNF gene expression in mice treated with 80 and 800 mg kg^{-1} of SWNTs or 80 mg kg^{-1} of MWNTs compared with NC mice. More surprisingly, BDNF gene expression was increased in mice treated with 800 mg kg^{-1} of MWNTs, the reason for which is not clear. We cautiously hypothesize this odd result as a case of hormesis because the doses employed were subtoxic, although a complete dose response study is needed to clarify this point. To support this hypothesis, *in vitro* toxicological studies found a hormetic dose–response following exposure to CNTs (for a review, see Iavicoli et al., 2010). One of the striking features of the present study was a significant decline of BDNF mRNA content in SWNT-treated groups compared with the group treated with 800 mg kg^{-1} of MWNTs. This finding further explains that (toxico)biological effects of CNTs are in line with their physicochemical characteristics. Several lines of research showed altered gene expression following exposure to CNTs (e.g. Pacurari et al., 2011). This study is the first to show altered BDNF gene expression after i.p. exposure to CNTs, particularly MWNTs.

Conclusion

The results of this study showed that CNTs could cause behavioral toxicity such as anxiety and depression that depended on their structure, as MWNTs were more toxic than SWNTs. The BDNF gene expression in the brain tissues of SWNTs-treated mice decreased at both low and high doses when compared to control mice. The BDNF gene expression decreased and increased in brain tissues of mice treated with low and high doses of MWNTs compared with control mice, respectively. Further investigations are needed to explain why BDNF gene expression changed following exposure to CNTs. We did not find

any correlations between ethological endpoints and BDNF mRNA and protein in the brain tissues of CNTs-treated mice. Therefore changes in the level of other biomolecules may be involved in behavioral changes that occurred following exposing to CNTs. We employed one single i.p. dose of CNTs in this study; further work will be required to determine if repeated and prolonged exposure to CNTs severely change behavioral and neurochemical profiles in the *in vivo* systems.

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