DOI: 10.4149/BLL_2023_106

Bratisl Med J 2023; 124 (9)
690 – 698

OBSERVATIONAL STUDY

Inhalation of multi-wall carbon nanotubes changes the expression of apoptosis and cancer genes in rat brain and lungs

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ABSTRACT
One of the important issues in urban areas is air pollution which causes respiratory disorders. A significant association between exposure to inhaled particulate matter (PM), mainly ultrafine particles, and increased neurological and pulmonary morbidity and mortality was observed in some research. This study aimed to demonstrate the relation between multi-wall carbon nanotubes (MWCNTs) inhalation and the carcinogenic effect of these materials in the brain and lungs. For this purpose, we investigated gene expression in rat brain and lung tissues induced by exposure to MWCNTs. Rats were exposed to MWCNTs in diameters of 10 and 100 nm (pure and impure) at a concentration of 5 mg/m^3. Exposure was done through a whole-body exposure chamber for 5 h/day, 5 days/week for 14 days. After exposure, both brain and lung tissues were isolated to evaluate certain gene expressions including *Bax, Bcl2, Rac1, Tp53, Mmp12*, and *Arc*. The results showed that exposure to impure and pure MWCNTs (10 and 100 nm) at a concentration of 5 mg/m^3 causes up-regulation or down-regulation of some of these genes. The results suggest that impure and pure MWCNTs (10 and 100 nm) can increase the risk of central nervous system disorders such as Alzheimer's disease and increase the risk of carcinogenesis in the lung tissues of rats exposed to MWCNTs *(Tab. 2, Fig. 2, Ref. 64)*.
Text in PDF *www.elis.sk*
KEY WORDS**:** multi-wall carbon nanotube, inhalation, gene expression, carcinogenicity, brain, lung.

Introduction

At the dawn of the 21st century, air pollution was one of the most important issues in urban areas which causes respiratory and cardiovascular disorders, neurological problems such as Parkinson's and Alzheimer's disease, strokes, and brain cancer (Ljubimova et al, 2018). Components in mixtures of air pollution, such as particulate matter, organic compounds, metals, and gases, can result in diseases. Our study has focused on carbon nanotube (CNT) as a particulate matter and the role of metal impurities in the toxicity of this nanomaterial. A significant association between exposure to inhaled particulate matter (PM), mainly ultrafine particles, and increased cardiovascular and pulmonary morbidity and mortality was observed in some research (Rückerl et al, 2011).

Nanoscale PM in urban traffic in Los Angeles caused inflammation and oxidative stress in the epithelium of the olfactory system and brain (Cheng et al, 2016). Also, it has been reported that extended exposures of rats to diesel exhaust result in neuroinflammation (Gerlofs-Nijland et al, 2010), as well as anxiety and increased formation of plaque in an Alzheimer›s disease model in the rat (Hullmann et al, 2017; Salvi et al, 2017).

Carbon nanotubes (CNTs) were discovered in 1991 by Iijima as materials with electrical, mechanical, and thermal properties (Iijima, 1991) applied in different biomedical and industrial usage. With the advancement of science and technology, these compounds are now more and more considered and studied (Jacobsen et al, 2017). Hundreds of tons of nanomaterials already enter the environment annually, but very little is known about the adverse effects of nanomaterials on biological systems (Drobne, 2007). Many studies show that these nanomaterials are toxic for organisms and especially for a human, and the presence of CNT in the environment modifies the physicochemical behavior of environmental pollutants, such as heavy metal ions, as reviewed by Rao et al. (Rao et al, 2007).

Combustion of fuel gas due to diesel-powered vehicles, automobiles, and industrial processes lead to production of MWCNTs in air borne particles in the environment. Because of large fuel gas

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Acknowledgements: This work was supported by the deputy of research of Shahid Beheshti University of Medical Sciences (Grant No. 10812).

consumption, MWCNT and air pollution have notable contribution (Lam et al, 2006).

Carbon nanotubes are fibrous and have similar properties to asbestos in terms of biological resistance (Soto et al, 2007; Poland et al, 2008; Sakamoto et al, 2009), and concerns have been raised about their potential adverse effects on human health and the environment (Ma-Hock et al, 2009). Because the size of the nanomaterial is comparable with some biological macromolecules such as antibodies, DNA, and enzymes, these materials were taken into consideration (Iijima, 1991). The diameter of CNTs can be a few nanometers, and their length is hundreds of microns. They have different structures, layers, lengths, and diameters (Hirlekar et al, 2009). Moreover, their uniqueness is due to a pattern of strong bonding between atoms and their excessive aspect ratio (Mohanta et al, 2019).

An important classification of CNTs includes single-walled carbon nanotubes (SWCNTs), including one graphene layer with a diameter of 1–2 nm and multi-walled carbon nanotubes (MWCNTs) consisting of tubes that were nested with a diameter more than SWCNTs (Hirlekar et al, 2009).

Nanoparticles may enter the body in various ways, such as inhalation, injection, dermal penetration, and ingestion, and may be distributed to various tissues by the circulatory system (Takenaka et al, 2001; Umeda et al, 2013). Human exposure to CNTs is primarily through inhalation contact (Johnston et al, 2010). During CNT manufacturing, exposure to dust or nanoparticles may occur and lead to inhalation of these nanotubes (Jacobsen et al, 2017; Kuempel et al, 2017). Thus it is essential to evaluate the toxicity of these nanomaterials because of their increased use (Wani et al, 2011) that lead to occupational, consumer and environmental exposure (Jacobsen et al, 2017).

Inhalation of CNT through the respiratory system is one of the important routes of exposure to these nanotubes (Manke et al, 2014). After inhalation exposure to CNTs, physicochemical properties such as particle diameter, size, and shape are important in the intensity of CNT toxicity (Wörle-Knirsch et al, 2006; Pulskamp et al, 2007). Because CNTs are not macromolecules, they settle as clumps in the inner walls, resulting in tumor inner walls of respiratory tracts (Porter et al, 2010; Polimeni et al, 2015).

The effect of CNTs on brain cells leads to release of some mediators from astrocytes and microglia that result in oxidative stress or apoptosis and inflammation (Bardi et al, 2013). Animal studies demonstrated that short-term pulmonary exposure to nanoparticles could induce an intensive inflammatory reaction in the lung relating to the physical and chemical properties of the nanomaterial tested (Kolling et al, 2011; Poulsen et al, 2015; Snyder-Talkington et al, 2016)

In general, despite numerous studies that reported the toxic effects of carbon nanotubes, there are still some uncertainties about the toxicity of these nanoparticles (Ema et al, 2016). To date, most toxicity studies have focused on inhalation exposure to CNT, and assessed its effect on cytotoxicity. In the current study, we aimed to investigate the risk of carcinogenesis of MWCNTs in rat lung and brain tissues by evaluating six specific apoptosis/cancerous-related gene expressions by quantitative real-time PCR (qPCR) following inhalation exposure of rats with these nanomaterials.

Materials and methods

Materials

Multi-wall carbon nanotubes (MWCNTs)

The MWCNTs were purchased with different characteristics from the Research Institute of the Petroleum Industry (RIPI) in Iran. We used MWCNTs with purity and impurity (containing Co, Fe, Mo, and Mg). This study evaluated and compared pure and impure MWCNTs with 10 nm and 100 nm diameters.

MWCNTs characterization

Structure characterization was evaluated with the sonication of MWCNTs in ethanol. To specify the diameter and length of MWCNTs fibers, transmission electron microscopy (HRTEM, LEO-912- AB; Carl Zeiss SMTAG, Oberkochen, Germany) was used. The MWCNTs' diameter and length were measured and displayed as a mean. MWCNTs diameter was the space between mean values of bright/dark contrast oscillation at the edges of tube walls (Samiei et al, 2020). Metal impurity content was identified by inductively coupled plasma-mass spectrometry (ICPMS, Perkin Elmer Elan6000, Waltham, MA) (Siegrist et al, 2014).

Dispersion of MWCNTs

Because of MWCNTs' hydrophobic properties and to improve the water solubility of MWCNTs, the dispersion protocol of nanogenotox was used (Jensen et al, 2011; Phuyal et al, 2017). Briefly, 2.56 mg/mL MWCNTs were wetted with 30 µL ethanol, then they were suspended in sterile-filtered 0.05 % bovine serum albumin (BSA) in MilliQ water, eventually sonicated for 16 min in an ice-water bath.

Animal exposure

The rats (Wister male) were purchased from Pasteur Institute, Tehran, Iran. Those weighing 180–220 g and 3–7 weeks old were selected. Rats were kept under controlled laboratory conditions (temperature of 25 ± 2 °C, humidity of 50–60 %, and a 12:12 hour light/dark cycle) with access to food and water. All experimental procedures were carried out by the ethical standards and protocols approved by the Animal Experimentation Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran, with IR.SBMU.RETECH.REC.1399.270 Ethical Committee code.

Studied animals were divided into five groups. Groups of 10 male rats in the chamber were exposed to either clean air (control) or different types of MWCNTs aerosol (10 nm Pure, 100 nm Pure, 10 nm Impure, and 100 nm Impure) at a concentration of 5 mg/m^3 for 5 hours a day, 5 days a week and for 2 weeks (Umeda et al, 2013).

Inhalation chamber

The chamber was 90–60–50 cm and made of polycarbonate plexiglass. The airflow rate of the chamber was 25 l/min, and the air circulation of the chamber was 56 l/min for 5 h. This chamber was planned to function with a standard two ventilators on

the roof, which maintains 10 rats in open mesh cages suspended above bedding material to decrease overcrowding and increase the free circulation of MWCNTs around the rats (Samiei et al, 2020).

In our study, we used exposure chambers for exposure to fresh air (as control or un-exposed group) and also for exposure to a different type of MWCNTs (5 mg/m^3) for 5 h/day, 5 days/week for 2 weeks (as an exposed group). An exposure concentration (5 mg/m3) of MWCNTs has been obtained based on our pilot study and previous studies (Umeda et al, 2013).

Fourteen days after the exposure, rats were euthanized, and their lungs and brains were harvested and immediately transferred to liquid nitrogen and used for experiments.

Real time PCR analysis

The collected lung and brain tissues were used to investigate molecular gene expression. Total RNA was purified using RNeasy Mini Plus Kit (Qiagen, Hilden, Germany) according to the manufacture's information. The extracted RNAs were stored at –70 ° C for subsequent steps (Lafzi et al, 2016). The high-capacity cDNA reverse transcription Kit (Qiagen, Hilden, Germany) was used for cDNA synthesis. cDNAs were examined by real-time PCR by the SYBR Green method. In this study, real-time qRT-PCR was used to evaluate and compare the effects of some types of MWCNTs (different in size and purity) on the mRNA expression of *Bax*, *Bcl2*, *Tp53*, *Mmp12*, *Rac1,* and *Arc* genes in the lung and brain tissues of exposed (test groups) and unexposed (control groups) rats. Gene expression was evaluated by $2^{-\Delta\Delta Ct}$ method and the Ct values of GAPDH gene expression were used for normalization (Tabatabaei et al, 2017). The following primers were utilized in real time PCR to measure the proposed gene expression (Tab. 1).

Statistical analysis

Three repetitions of all tests were done. The gene expression analysis results were analyzed with Graph Pad Prism software, version 8.07 (Graph Pad Software, Inc., La Jolla, USA) using one-way ANOVA analysis followed by Tukey's post hoc test for the determination of differences between the mean values. The data were displayed as the mean ± standard deviation (SD). $p < 0.05$ was considered to show a statistically significant difference.

Results

MWCNTs characterization

The diameter and length of MWCNTs are displayed in Figure 1 and Table 2.

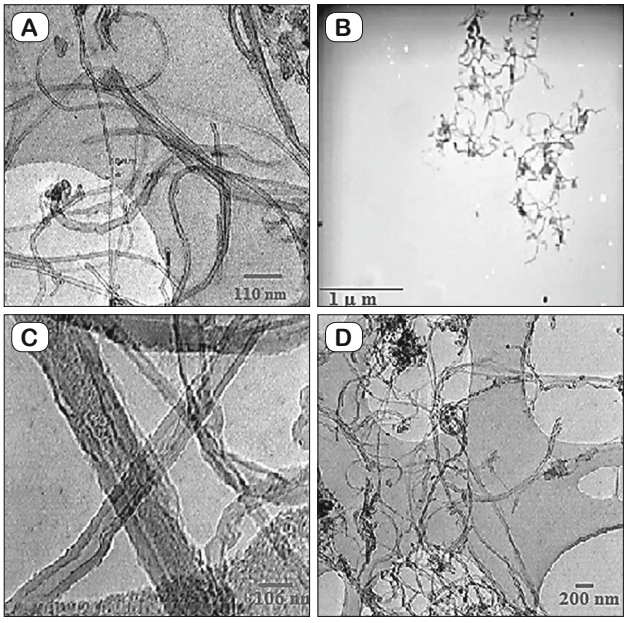

Fig. 1. TEM image of MWCNTs. A) 10 nm Pure MWCNTs, B) 10 nm Impure MWCNTs, C) 100 nm Pure MWCNTs, D) 100 nm Impure MWCNTs. TEM: transmission electron microscopy; MWCNTs: multi walled carbon nanotubes.

Gene expression analysis

Bax gene expression

As shown in Figure 2, 10 nm carbon nanotubes (CNTs) (pure and impure) caused significant up-regulation of the *Bax* gene compared to the control group (unexposed to CNTs; gene expression level in the tissue is equal to 1) in brain tissue (approximately 2-fold). However, the difference in gene expression between pure and impure groups was not statistically significant ($p < 0.05$). *Bax* gene expression in 100 nm pure and impure CNTs did not show a significant difference compared to the control group (expression level is almost equal to control) and relative to each other ($p < 0.05$). Up-regulation of *the Bax* gene (approximately 2-fold) in the 10 nm pure CNTs is significant ($p < 0.05$) in brain tissue compared with the 100 nm pure carbon nanotubes group. Similarly, the *Bax* gene expression in the 10 nm impure CNT was significantly higher than the 100 nm CNT (about 2-fold).

In lung tissue, up-regulation of *the Bax* gene in all four groups exposed to CNTs increased significantly ($p < 0.05$) compared to the control group (Fig. 2a). Up-regulations of the *Bax* gene in 10 and 100 nm pure CNTs were about 22 and 75-fold, and in the 10 and 100 nm, impure CNTs were about 184 and 228-fold, respectively. The gene expression level in the 10 nm impure CNT group was significantly higher (approximately 8-fold) than in the pure group at the same size (10 nm). On the other hand, increased expression in the 100 nm impure CNTs was significant in comparison to the pure group at the same

Tab. 1. MWCNTs characterization. The diameter and length of MWCNTs.

MWCNTs properties	Diameter (nm)	Length (mm)	Impurities as mass %
10 nm Pure MWCNTs	10	0.14–1.7	
10 nm Impure MWCNTs	10	0.14–1.7	Co (0.12), Fe (0.16), Mo (0.12), Mg (0.13)
100 nm Pure MWCNTs	100	0.16–1.8	
100 nm Impure MWCNTs	100	0.16–1.8	Co (0.12), Fe (0.16), Mo (0.12), Mg (0.13)

Tab. 2. Specific primers for assessing the expression of *Bax, Bcl2, Rac1, Tp53, Mmp12* and *Arc* genes.

Gene Symbol (Full name) Gene Bank Refseq (mRNA)	Sequence (5' ->3')	Product Length (bp)
Arc (activity-regulated cytoskeleton-associated protein) NM_019361.1	F: CCCTGCAGCCCAAGTTCAAG R: GAAGGCTCAGCTGCCTGCTC	115
Rac1 (Rac family small GTPase 1) (Ras-related) NM_134366.1	F: CAATGCGTTCCCTGGAGAGT R: AGAACACGTCTGTTTGCGGG	161
Bax (BCL2 associated X, apoptosis regulator) NM_017059.2	F: ACCAAGAAGCTGAGCGAGTG R: TCCACATCAGCAATCATCCTCT	86
Bcl2 (BCL2, apoptosis regulator) NM_016993.1	F: CTGCACCTGACGCCCTTCACC R: CACATGACCCCACCGAACTCAAAGA	119
Tp53 (tumor protein p53) NM_030989.3	F: CAGCTTTGAGGTTCGTGTTTGT R: ATGCTCTTCTTTTTTGCGGAAA	82
Mmp12 (matrix metallopeptidase 12) NM_053963.2	F: GCATTCAGTCCCTCTACGGAGC R: AAGTAATGTTGGTGGCTGGACT	189
Gapdh (glyceraldehyde-3-phosphate dehydrogenase) NM_017008.4	F: AGTGCCAGCCTCGTCTCATA R: AGAGAAGGCAGCCCTGGTAA	92

size (approximately 3-fold). *Bax* gene expression differences in pure but not in impure 10 nm and 100 nm CNT exposure groups were significant ($p < 0.05$).

Bcl2 gene expression

As seen in Fig. 2b, the expression of the *Bcl2* gene in the brain tissue of rats in groups exposed to 10 and 100 nm pure CNTs was not significantly different from that of the control group (unexposed to CNTs) ($p < 0.05$). Significant down-regulation was noted in the expression of *Bcl2* gene expression in groups exposed to 10 and 100 nm impure CNTs (about a 3 and 8-fold decrease, respectively) ($p < 0.05$) compared with the control group. However, there was no significant difference in expression levels between these two impure CNT groups. The comparison of reduced *Bcl2* gene expression (less than half) between impure and pure CNTs (with the same diameter) in exposed groups was also statistically significant ($p < 0.05$).

According to the results (Fig. 2b), the CNTs exposure led to significant up-regulation of *the Bcl2* gene in all four lung tissue groups ($p < 0.05$) compared to the control group (unexposed to CNTs). Up-regulation of *the Bcl2* gene in 10 and 100 nm pure CNTs was about 27 and 35-fold, respectively, but it was about 117 and 55-fold, in the same diameter impure CNTs. Expression of the *Bcl2* gene in the 10 nm impure CNT group was significantly higher (approximately 4-fold) than in the pure (10 nm) group ($p < 0.05$). However, up-regulation of *the Bcl2* gene in the 100 nm impure CNTs was not statistically significant ($p < 0.05$) compared to the 100 nm pure group. *Bcl2* gene expression in groups exposed to impure 10 nm CNTs was significantly higher than in those exposed to 100 nm impure carbon nanotubes ($p < 0.05$) but not significant in pure forms (in two different sizes) ($p < 0.05$).

The *Bax/Bcl2* expression ratio in brain tissue after exposure to 10 nm pure and impure MWCNTs has been increased 2 and 2.6-fold, and following exposure to 100 nm pure and impure MWCNTs has been increased 1.4 and 6.5-fold. The expression ratio of *Bax/Bcl2* in lung tissue following exposure to 10 nm impure MWCNTs has been increased 1.6-fold and following 100 nm pure and impure MWCNTs have been increased 2.1 and 4.1-fold, respectively.

Rac1 gene expression

The results showed that in the brain tissue, 10 nm CNTs (pure and impure) significantly up-regulated *Rac1* gene expression compared to the control (~2-fold) ($p < 0.05$). *Rac1* gene expression in the groups exposed to 100 nm pure and impure CNTs (without significant differences) showed no significant differences ($p < 0.05$) compared to the control group (Fig. 2C).

Significant up-regulation was noted in the expression of *Rac1* in the groups exposed to 10 nm impure CNTs compared with 100 nm impure CNTs (approximately 2-fold) ($p < 0.05$), as well as in pure groups.

As can be seen in Fig. 2C, only the 10 nm impure CNTs group leads to significant up-regulation of the *Rac1* gene (approximately 5-fold) in lung tissue compared to the control group ($p < 0.05$). Increased expression of *Rac1* gene with 10 nm impure CNTs group was also significantly higher than the 10 nm pure CNTs (no significant difference compared to the control) and 100 nm impure carbon nanotubes groups. The difference between the *Rac1* gene expression in 100 nm pure and impure groups (without significant differences) was not statistically significant compared with the control group ($p < 0.05$).

Tp53 gene expression

In brain tissue, all four groups of MWCNTs caused significant up-regulation of the *Tp53* gene ($p < 0.05$) compared to the control group. This increase is approximately 3.5-fold in the 10 nm pure and 100 nm pure and impure CNT groups and about 6-fold in the 10 nm impure CNT group (Fig. 2D). Also, up-regulation in the 10 nm impure CNT group was approximately 1.5-fold higher than the 10 nm pure CNT group.

According to the results, exposure to all four groups of CNTs significantly led to up-regulation of the *Tp53* gene in the lung tissue compared to the control group ($p < 0.05$). This increase is about 3-fold in 10 and 100 nm pure carbon nanotube groups and about

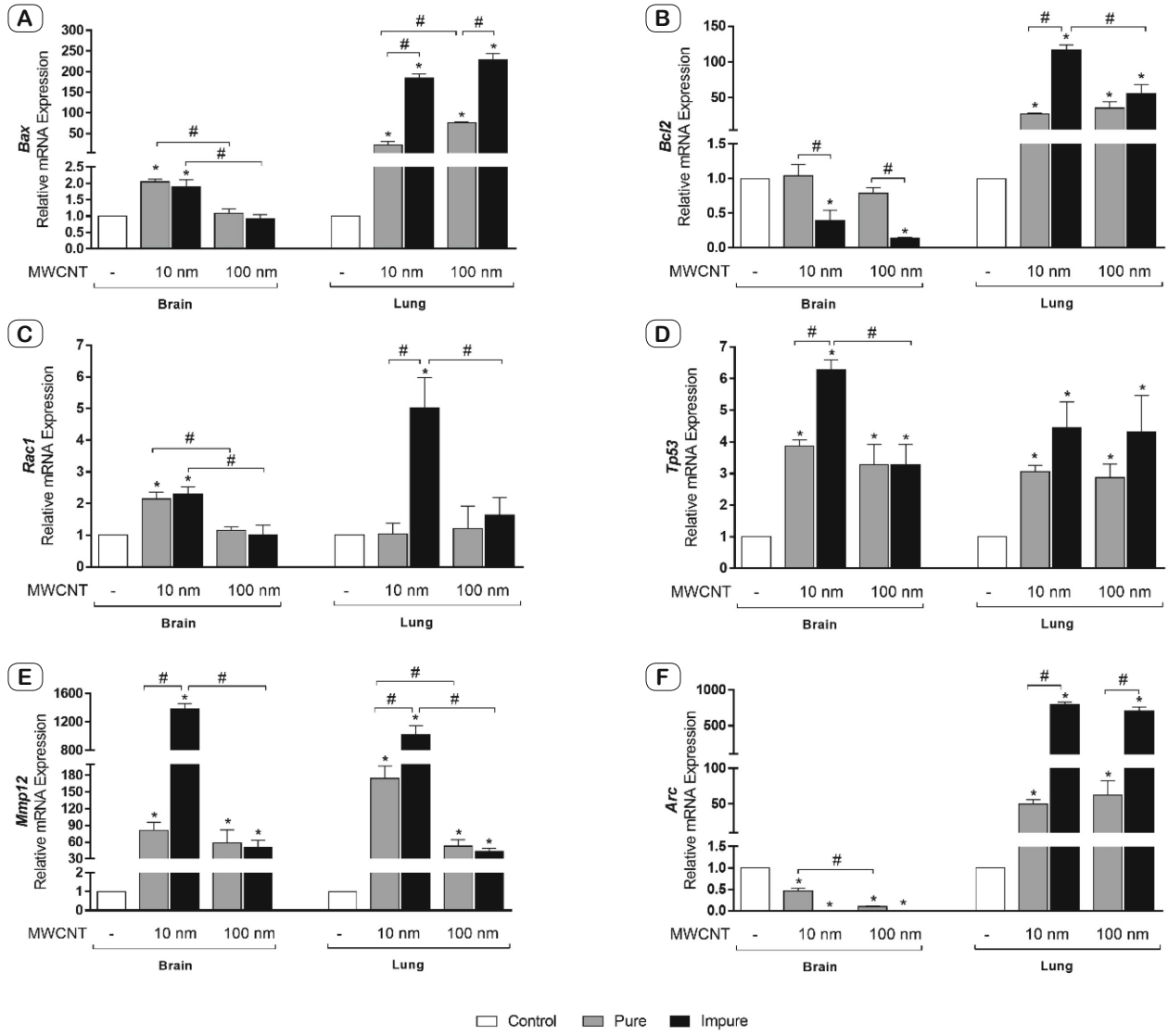

Fig. 2. Quantitative analysis of the *Bax* (inducing apoptosis) (A), *Bcl2* (Anti apoptosis) (B), *Rac1* (Oncogene) (C), *Tp53* (Tumor suppressor) (D), *Mmp12* (Macrophage Metallo elastase - involved in tissue damage, metastasis, emphysema, etc.)(E), and *Arc* (Involved in the formation of memory, learning, cell migration, immune regulation, cell morphogenesis regulation, cellular skeleton formation and ...) gene expression in brain and lung tissues of *Rattus norvegicus* after two weeks without exposure (control group with the gene expression equal to 1) and with inhalation exposure to 10 and 100 nm, pure and impure multi wall carbon nanotubes for 2 weeks. Data are presented as mean ± SD (n = 3).The one-way ANOVA test (Tukey's post hoc test) carried out. (*) shows a significant difference (decrease or increase) in comparison with the control group (* $p < 0.05$). (#) on the line between the two columns shows a significant difference (decrease or increase) between the two groups ($^{\#} p < 0.05$).

4.5-fold in 10 and 100 nm impure carbon nanotube groups. No significant change was noted in the expression of the *Tp53* gene between pure and impure groups in both sizes ($p < 0.05$). Also, the difference in expression between two pure groups in two different sizes (relative to each other) and also in impure groups was not significant ($p < 0.05$).

Mmp12 gene expression

According to the results in Figure 2E, a significant increase in *Mmp12* gene expression in the brain tissue was seen in 10 nm pure and impure (about 81 and 1380-fold, respectively) and also in 100 nm pure and impure (about 59 and 51-fold; without significant difference between them) CNT groups ($p < 0.05$). Up-regulation of *the Mmp12* gene with 10 nm impure CNTs was 17-fold higher than 10 nm pure and 28-fold higher than 100 nm pure and impure CNT groups.

As shown in Figure 2E, in all four groups exposed to CNTs, significant up-regulation of the *Mmp12* gene was detected in lung tissue. This significant up-regulation was about 174 and 53-fold in 10 and 100 nm pure, respectively, and about 1000 and 43-fold in

10 and 100 nm impure CNTs groups. *Mmp12* gene up-regulation in the 10 nm impure CNT group was more significant than that of the pure group of the same size (approximately 5-fold). However, the difference between 100 nm pure and impure groups was not statistically significant ($p < 0.05$). There was also a significant difference between the 10 and 100 nm pure CNT groups and between the 10 and 100 nm impure groups ($p < 0.05$).

Arc gene expression

As seen in Figure. 2F, all four groups of CNTs significantly caused downregulation of *the Arc* gene in the brain tissue ($p < 0.05$) compared to the control group. The decreased expression level in pure 10 and 100 nm CNTs was about half and one-tenth of the unexposed control group, respectively. Interestingly, down-regulation of the *Arc* gene in groups exposed to impure 10 and 100 nm carbon nanotubes was measured near zero ($p < 0.05$). The expression level of *the Arc* gene in the group exposed to 100 nm pure CNTs was significantly lower (approximately one quarter) than in the group exposed to the 10 nm pure CNTs.

Interestingly, in the lung tissue, a significant increase was seen in all CNT groups ($p < 0.05$) (Fig. 2F). This up-regulation was 50 and 62-fold in 10 and 100 nm pure CNTs and 793 and 702-fold in 10 and 100 nm impure CNT groups. Significant differences were noted in the expression of *the Arc* gene between 10 nm pure and impure CNTs and between 100 nm pure and impure CNTs.

Discussion

Because of the increasing use of MWCNT in industry, occupational exposure of workers causes concern. Workers, lab researchers, and consumers with long-term CNTs exposure are at risk of the adverse effect of these nanomaterials (Gangoli et al, 2019; Chakraborty et al, 2018; Pelin et al, 2018) and also because of the large consumption of fuel gas which causes to produce MWCNTs in airborne particle in the environment, MWCNT and air pollution have notable correlation (Lam et al, 2006). In the present study, male rats were used to investigate the ability of two types of MWCNTs (pure and impure) with two diameter sizes (10 nm and 100 nm) to change specific gene expression in brain and lung tissues following inhalation exposures and clarify the underlying potential carcinogenesis caused by these nanomaterials. After two weeks of exposure, evaluation for quantitative gene expression was done.

For this aim, we examined *Bax* (apoptosis inducer), *Bcl2* (Anti apoptosis), *Rac1* (Oncogene), *Tp53* (Tumor suppressor), *Mmp12* (metastasis inducer- involved in tissue damage, emphysema, etc.), and *Arc* (Involved in memory formation, learning, cell migration, immune response regulation, cell morphogenesis regulation, cellular skeleton formation, etc.) gene expression in brain and lung tissues of rats which were exposed to these specific types of MWCNTs using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR).

A novel finding which might lead to a better understanding of the carcinogenicity risk of MWCNTs in the brain and lung tissues has resulted after two weeks of inhalation exposure of rats to these nanotubes.

Bax and *Bcl2* are necessary for apoptosis regulation. Our results showed that *Bax* was up-regulated after exposure to 10 nm pure and impure CNTs in rat brain tissue. Also, *Bax* has been significantly up-regulated in lung tissues following CNTs exposure, especially impure MWCNTs. These results are in agreement with the previous study, which reported that MWCNTs induced significant up-regulation of *Bax* and *P53* in lung cells (Srivastava et al, 2011).

Bcl2, an anti-apoptotic gene, prevents apoptosis or programmed cell death and advances cell survival by suppressing *Bax* function (Hara et al, 1997). Our results showed down-regulation of *Bcl2* in brain tissues following CNTs exposure, especially impure MWCNTs. *Bcl2* in lung tissue has been up-regulated following exposure to MWCNTs, particularly to 10 nm impure one, which is in line with the previous study that reported that when *Bax* increases apoptosis of cancer cells, the *Bcl2* blocks the cell death pathway associated with *Tp53* (Srivastava et al, 2011).

Tp53, the tumor suppressor gene (Toyokuni, 2013a), is a cell cycle key regulator (Stark et al, 2007) that becomes inactive in human cancers and plays an important role in brain and lung cancer and cancer-prone hereditary disease (Toyokuni, 2013a). In our study, *Tp53* has been up-regulated in brain tissues after CNTs exposure, especially 10 nm impure MWCNTs, and also the result showed *Tp53* up-regulation in lung tissues following CNTs exposure. These findings are in line with the results of previous studies, which reported that intraperitoneal injection of MWCNTs leads to malignant mesothelioma in *p53* hetero-knockout Mice (Takagi et al, 2008).

Rac1 (a subfamily of the Rho family of GTPases) is a key gene in mediating active forgetting (Shuai et al, 2010).

The Kitamura et al. study in 2017 indicated that increasing *Rac1 is* associated with Alzheimer's disease (AD)-related memory loss, and inhibition of *Rac1* leads to an impressive therapeutic approach for AD (Kitamura et al, 2017). This finding is in agreement with our results, indicating that *Rac1* has been up regulated in brain tissues following 10 nm pure and impure CNTs.

The hippocampus is a very crucial region of the brain for recent memory formation (Kitamura et al, 2017) and hence is more related to primary signs and symptoms of memory loss in AD patients (Mufson et al, 2015). It is considered that in the hippocampus, daily episodic memory and recent spatial memory are organized and saved and then transfer of memory to the prefrontal cortex occurs (Kitamura et al, 2017), which is in line with our previous study that revealed ROS generation and apoptosis in the hippocampus as well as frontal cortex (Samiei et al, 2020).

The *Rac1* gene was up-regulated after exposure to 10 nm impure MWCNTs in lung tissues. This finding is in agreement with previous studies that demonstrated *Rac1* is one of the important prognostic lung cancer markers and stimulates invasion, metastasis, progression, or recurrence of lung cancer (Zhou et al, 2016). These data favor the hypothesis that *Rac1* up-regulation may be necessary for lung tumor development.

Tumor invasion and angiogenesis need to control extracellular matrix component destruction to permit tissue formation and cell migration. As a zinc-dependent endopeptidase, matrix metal-

loproteinase (MMP) plays a substantial role in these processes (Kachra et al, 1999).

MMP genes are implicated in neuro-inflammatory CNS disorders including Parkinson's disease (PD), Alzheimer's disease (AD), stroke, multiple sclerosis (MS), epilepsy, and cerebral aneurysm (Rempe et al, 2016).

Several studies observed up-regulation of *MMPs* in rodent AD models compared to control animals (Rempe et al, 2016). Also, our results have shown *Mmp12* up-regulation following MWCNTs, especially the 10 nm impure one.

Our data demonstrated *Mmp12* up-regulation in lung tissue following MWCNTs, specifically 10 nm impure, which agrees with some studies. For example, a study reported a 13.59-fold increase in *Mmp12* mRNA levels in lung alveolar epithelial cells after exposure to MWCNTs, which is certainly in agreement with our study (Pacurari et al, 2017).

A study with knock-down *Arc* showed that genes involved in memory and learning processes and genes implicated in synaptic plasticity (Bloom, 2014) were down-regulated after decreasing *Arc* expression. These genes play a critical role in the growth and development of dendrites and axons. Some synaptic genes implicate neuroplasticity, memory, cognition, and learning. *Arc* leads to changes in gene expression, which has a role in the neurotoxicity and neurodegeneration of AD, such as *Bcl2 l11*, which is in agreement with our finding that demonstrates the down regulation of *Bcl2* in the brain. Knock-down of *Arc* resulted in the generation and accumulation of amyloid-beta (Aβ) and formation of neurofibrillary tangles (NFTs), which are hallmarks of AD. This information shows an important role of *Arc* in AD pathophysiology (Bloom, 2014).

In addition, *Arc* knock-down has a relationship with psychological disorders, such as basal ganglia disorder, Huntington's disease, Alzheimer's disease, central nervous system (CNS) amyloidosis, and tauopathy, which among these, tauopathy and CNS amyloidosis are predictors of AD. These evidences suggest that the *Arc* function has a significant role in modulating these disorders' progression (Leung et al, 2019).

In this study, *Arc* was down regulated in brain tissues after exposure mostly to Impure MWCNTs, which is in agreement with previous studies that suggested the decreased *Arc* gene expression in mammalian brain tissue is an indicator of increased risk of Alzheimer's disease.

On the other hand, *Arc's* relevance with a c-fos oncogene, a Ras oncogene family member (Okuno, 2011; Hossaini et al, 2010), and its correlation with tumor growth and changes in the cytoskeleton could predispose the spread and development of tumors (Leng et al, 2015).

One report observed significant up-regulation of *Arc* in human lung cancer cells A549, SPC-A1, and H322 compared with that of normal lung cells MRC-5 (Guoli et al.), which is in line with our results that showed up-regulation of the *Arc* gene in lung tissue following exposure to CNTs, especially to impure MWCNTs which may be an indicator of increased risk of carcinogenesis.

We have identified for the first-time relevant changes in the expression of specific genes, such as *Arc* and *Rac1,* in rats exposed to MWCNTs. *Rac1* and *Arc* are specific genes that could play essential roles in tumor progression and memory and have a critical role in neurodegenerative diseases such as Alzheimer's (Bardi et al, 2013).

Some hypotheses propose that the physical properties of CNTs are responsible for pulmonary toxicity (Manke et al, 2014). Physiochemical properties of CNTs associated with respiratory toxicity and carcinogenicity after inhalation exposure include length, diameter, surface modification, and rigidity of MWCNTs (Mohanta et al, 2019; Toyokuni, 2013b).

The current results show that the adverse effects of 10 nm MWCNTs were greater than those of 100 nm. These findings are in agreement with previous studies. For example, it has been reported that MWCNTs with a diameter of 145 nm were less pathogenic than those with a diameter of 50 nm (Nagai et al, 2011). One report demonstrated a relationship between the MWCNTs diameter and alveolar macrophage toxicity, inducing MWCNTs that the thinner MWCNTs were more toxic (Fenoglio et al, 2012). Another report demonstrates a relationship between the MWCNTs diameter and the alveolar damage in *p53* hetero-knockout mice (Takagi et al, 2012).

Regardless of genetic toxicity data, the data display that all MWCNT types in this study can induce a considerable alteration in gene expression in brain tissues that leads to neurological disorders and also cause the expression of significant genes involved in pathways of carcinogenesis in lung tissues.

It could be hypothesized that MWCNTs would extend into neurological diseases in the brain and carcinogenicity in the lung after a two-week inhalation exposure period. Our data propose that more research is required to recognize the *in vivo* genotoxic potency of MWCNTs.

According to the results, it may be interpreted that some types of MWCNTs can change specific gene expressions; in which various factors such as CNTs diameter, CNT purity, rat strain, duration of exposure, and post-exposure time to start of gene expression examining could have a considerable effect on final results.

The data presented in our study shows that inhalation exposure of rats to MWCNTs, as an important product of fuel gas consumption, especially to 10 nm impure MWCNTs, induces specific gene expression changes in the brain and lung tissues. Some of these changes are associated with neurodegenerative diseases such as Alzheimer's and lung tumor development. Our findings suggest the possible appearance of some critical disorders in the brain and premalignant lung changes after exposure to certain types of MWCNTs. Ten nm Impure MWCNTs could play a significant role in gene expression changes in the brain and lungs.

Conclusion

Our comparative analysis of gene expression shows that inhalation exposure of rats to MWCNTS, especially 10 nm impure ones at the concentrations studied, is associated with lung tumor development. Significant changes were observed in the regulation of some genes such as pro- and anti-apoptotic genes, oncogenes, tumor suppressor genes, and those genes involved in metastasis,

cell migration, immune regulation, cell morphogenesis regulation, cellular skeleton formation in brain and lung tissues and the formation of memory and learning in brain tissues exposed to CNTs. Considering this study's limitations, further research with a larger sampling of rats is needed.

According to our findings of specific gene expression in brain tissues of exposed rats, it is suggested that MWCNTs can increase the risk of a neurological disorder such as Alzheimer's disease. On the other hand, our results of gene expression demonstrated that MWCNTs could increase the risk of carcinogenesis in the lung tissues of exposed rats.

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Received March 2, 2023.
Accepted April 14, 2023.