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Research Article

Evaluation of *TNF* Family Gene Expression under the Influence of Single-Walled and Multi-Walled Carboxylated Carbon Nanotubes in Jurkat Cell Line and Rat

Shirin Lotfipanah[1], Parichehreh Yaghmaei[1*], Majid Zeinali[2], Seyed Ali Haeri Rohani[1], Sosan Kabodanian Ardestani[3]

[1] Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2] Biotechnology Research Center, Research Institute of Petroleum Industry (RIPI), Tehran, Iran.
[3] Institute of Biochemistry and Biophysics, Research institute in Tehran University, Tehran, Iran.

**Corresponding author:* Parichehreh Yaghmaei, Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
Tel/ Fax: +98- 2122363520; E-mail: yaghmaei_p@srbiau.ac.ir

Background: Nanomaterials, e.g.carbon nanotubes (CNTs), have broad usage in medicine for diagnosis, treatment, and drug delivery. Prior to the widespread use of CNTs, any potential toxicity issues must be considered. Apoptosis is an important issue in toxicological studies, and tumor necrosis factor (TNF) family members execute crucial roles in apoptosis and inflammation. We examined the survival of Jurkat cells under the influence of single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs) as well as their impacts on the mRNA levels of TNF family transcripts in Jurkat cells and rats.

Objective: To evaluate the toxicity or safety of a specific concentration and form of CNT on the expression of one of the gene families of the apoptotic pathway.

Materials and Methods: Jurkat cells were exposed to SWCNTs and MWCNTs in carboxylated form ($SWCNT_S$-COOH and MWCNTs-COOH). MTT assay assessed the cell survival, and using qRT-PCR, the expression levels of *TNF*, *CD40LG*, *TNFSF10*, *TNFSF8*, *CD40*, *TNFRSF10A*, *TNFRSF10B*, *TNFRSF11B*, *TNFRSF1A*, *TNFRSF21*, *TNFRSF25*, and *TNFRSF9* were examined. The housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase was utilized for normalization. We also evaluated the expression levels of *TNF* and *TNFRSF10A* in rats *in vivo* 30 and 60 days after being injected with CNTs.

Results: After 72 h of carboxylated CNTs at 100 µg. mL^{-1}, no significant change was observed in the survival rate of treated Jurkat cells. The expression of two genes (*TNF* and *TNFRSF10A*) changed significantly. Examining the expression profiles of these two genes in rats demonstrated an insignificant change in the expression of any of these genes after 30 and 60 days. The qRT-PCR analysis exhibited the elevated levels of *TNF* and *TNFRSF10A* mRNA in the CNT-treated cells, while expression of other *TNF* family members did not significantly differ from control (untreated) Jurkat cells. There was also no significant change in the gene expression levels of *TNF* and *TNFRSF10A* in CNT-treated rats after 30 and 60 days.

Conclusions: Administration of SWCNTs-COOH and MWCNTs-COOH could result in the up-regulation of *TNF* and *TNFRSF10A* but did not initiate apoptosis in Jurkat cells. Carboxylated SWCNTs showed more potent activity than MWCNTs in activating *TNF* gene expression and probably trigger cell death through external apoptotic pathways.

Keywords: CNT_S; Gene Expression; Jurkat Cell; TNF Family

1. Background

Nanotechnology is the change of materials at the nanometer scale with the aim of producing products with unique features. Many nanomaterials are featured with new biological and chemical attributes that often do not occur naturally. Carbon nanotubes (CNTs) are man-made nanomaterials (1, 2) that possess special physical and chemical features and have wide applications in modern science and technology (2, 3). Uncertainty about the possible risks of CNT on the environment and human health is an important issue.

Over recent years, the focus of many research communities has been on nanomaterials such as fullerene derivatives, nanotubes, and nanowires that are used in the manufacture of biotechnological tools (4). CNTs can be found in various forms and can be

chemically or functionally modified by hydroxyl or carboxyl groups or even other nanomaterials (5). Single-walled CNTs (SWCNTs) in pristine form are basically sheets of graphene (with diameters ranging from 0.4 nm to micrometers) rolled up into a seamless cylinder. SWCNTs are tubular graphitic nanostructures of approximately 1.0 nm in diameter and have a variety of electronic features, depending on how the graphene sheet is rolled into a cylinder, known as chirality (6-8). Multi-walled CNTs (MWCNTs) are comprised of multiple SWCNTs stacked one inside another, and their diameters can range up to 100 nm.

Apoptosis generally occurs through the intrinsic and extrinsic pathways. The former is normally triggered in response to some cellular stresses. Interaction between the members of proapoptosis and antiapoptosis of the lymphoma 2 leukemia protein family initiates and controls the intrinsic pathway of apoptosis, thereby promoting the release of apoptotic inducers from mitochondria. The extrinsic pathway is induced by binding ligand to death receptors, a member of the tumor necrosis factor (TNF) receptor superfamily identified by cysteine-rich domains (9-11). All death receptors consists of a conserved cytoplasmic polypeptide that contributes to the cognition of apoptotic machinery (12).

Until date, various research works have addressed CNT toxicity in mice (12)and CNT cellular toxicity in apoptotic and necrotic *in vivo* and *in vitro* conditions (13-15). There are also some reports on the therapeutic effects of nanoparticles and TNF-related apoptosis-inducing ligand (TRAIL), a death ligand belonging to the TNF superfamily (17, 18). The gene expression of apoptotic pathway in Jurkat cells under the treatment of different substances has already been studied (19), and evidence has revealed that nanoparticle dimensions have a link with cytotoxicity (20, 21).

Notwithstanding the extensive applications of nanomaterials, little is known regarding the impacts of these materials on human health and the environment. In this light, this study examined the survival of Jurkat cells following exposure to MWCNTs and SWCNTs. In addition, we assessed the mRNA levels of TNF superfamily members in Jurkat cells before and after treatment in rats.

2. Objectives

Due to the widespread use of nanoparticles, especially in the medical sciences, it is necessary to investigate the toxicity or safety of these substances, and since one of the results of toxicity of these substances is apoptosis, in the present study at the level of gene expression and comparatively under conditions in vitro and in vivo, the expression level of one of the gene groups in this pathway(*TNF* family gene) under the influence of a specific concentration and form of carbon nanotubes is examined.

3. Materials and Methods

3.1. Materials and Instruments

The 3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and L-glutamine were acquired from Sigma Company (St. Louis, MO, USA). Nitric and sulfuric acid, tetrahydrofuran, and thionyl chloride were purchased from Acros Organics (Morris Plains, NJ, USA) and fetal bovine serum (FBS) and RPMI 1640 from Biosera (Nuaillé, France). Streptomycin-penicillin and the Jurkat cell line were obtained from Gibco (Gaithersburg, MD, USA) and the National Center for Genetic and Biological Resources of Iran (Tehran, Iran), respectively. Crude SWCNTs and MWCNTs were made at the Petroleum Industry Research Institute (RIPI, Tehran, Iran). The HPLC reagents and solvents or those of analytical grade used in this study were procured from Merck Millipore (Burlington, MA, USA). The kit of RNA extraction was from Bio Basic Inc. (Markham, Canada) and the kits of cDNA synthesis and SYBR Green Master Mix from Takara Bio Inc. (Kyoto, Japan).

3.2. CNT Functionalization

Following the dispersion of CNTs in the concentrated sulfuric acid:nitric acid (3:1), the sonication of mixture was performed using an ultrasonication bath (Elmasonic P, Germany) at the temperature of 40 ºC for a period of 4 h. Subsequently, CNTs were filtered via a PTFE membrane filter (0.2-mm diameter) and rinsed extensively with deionized water. Finally, FTIR spectroscopy verified the accuracy of CNT functionalization. After dispersing in phosphate-buffered saline (PBS, pH 7.4), Q5 carboxylated CNTs were autoclaved for further cellular assays (22).

3.3. Cell Culture

Jurkat (ATCC Catalog No. TIB-152), Tthe human T lymphocyte cell line, was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). After culturing in RPMI 1640 containing heat-inactivated FBS (10%), antibiotic (0.1 mg. mL^{-1} streptomycin and 100 IU. mL^{-1} penicillin), and L-glutamine (0.3 mg. mL^{-1}), Jurkat cells were incubated at the temperature of 37 °C in a CO_2 incubator with the humidity of 5%. The cells were then seeded at a density of 2×10^5 cell. mL^{-1} on

96- and/or 6-well plates for subsequent experiments. To determine the cell viability, the cultures were harvested, and live cells were counted by the Trypan blue staining using a standard hemocytometer.

3.4. Cell Treatments

Following the cell confluency reached 70%, the cells were washed with PBS. Subsequently, the cells were seeded (2×10^5 cells. mL^{-1}) on 24-well flat-bottom plates (Nunc, Roskilde, Denmark) and exposed to the preparation of carboxylated SWCNT or MWCNT (dispersed in PBS; 100 µg. mL^{-1}) for a period of 72 h. After culturing the cells for 48 h, the mRNA levels of various transcripts were evaluated.

3.5. Assessment of Cell Viability by MTT Assay

Cells were first seeded (1×10^4 cell/well) on flat-bottom microtiter plates of 96 wells and then allowed for adherence to a CO_2 incubator at the temperature of 37 °C for a period of 24 h. Following the replacement of the culture medium with a fresh one, the cell treatment was conducted with CNTs (100 µg.mL^{-1}) in the same incubator and temperature mentioned above. After 24 h and the replacement of culture medium with a new one, MTT working solution (10 µL; 5 mg/mL in PBS) was transferred to each well. Afterwards, the incubation (in CO_2 incubator, at 37 °C, for 4 h) of plate was accomplished. Aspiration of medium and solubilization of formed formazan crystals were performed by the addition of 50 µL of dimethyl sulfoxide per well and incubation at 37 °C for 30 min. In the end, using a microplate reader, the intensity of the dissolved formazan crystals was measured at 540 nm (23-25).

3.6. Treatment of Rats with Nanoparticles (MWCNT and SWCNT)

Male Wistar rats (n = 24) were procured from Razi Vaccine and Serum Research Institute, Tehran, Iran. All the rats were kept in an animal center in Biochemistry and Biophysics, Tehran University, Iran under controlled temperature conditions (22 ± 2 °C) with constant light of 12 hours (08:00–20:00). Three weeks before experiments, animals were randomly allotted to three equal groups. The first and second groups were treated with MWCNT (0.4 mg.mL^{-1}) and SWCNT (0.5 mg/rat), respectively, and the third group was treated with PBS as a control. The injection method was intraperitoneally. Animals were maintained in the animal center addressed above under standard conditions with *ad libitum* access to water and food. All the rats were housed at 20 °C with a constant photoperiod of 12 h. The protocol for animal experiments and care and use were approved by the Animal Ethics Committee of the University of Tehran, Iran. After 30 and 60 days, the whole blood (2 mL) was collected from all the animals. In the final step, Ficoll density gradient centrifugation (Sigma, Germany) was employed for the isolation of the peripheral mononuclear cells (PBMCs) isolated and for the extraction of total RNA.

3.7. RNA Extraction and cDNA Synthesis

Ths isolation of total RNA from Jurkat cells and rat PBMCs were carried out using QIAzol Lysis Reagent. The extracted RNA was assessed in terms of quality by measuring absorbance at 260/280 nm and by electrophoresis on agarose gels containing ethidium bromide. Complementary DNA was synthesized using a kit from Qiagen (the Quanti Tect Reverse Transcription Kit; Hilden, Germany). Using SYBR green Master mix, appropriate primers (2 pg.mL^{-1}), cDNA (200 ng), real-time PCR was performed. The following program was executed on a BIO-RAD CFX96 system (Bio-Rad, USA) for PCR amplification: at first, one cycle (95 °C for 15 min) and then 40 cycles (95 °C, 60 °C, and 72 °C for 30 s for each temperature). Real-time PCR reactions were conducted in triplicate. The quality of graphs and melting curves and the quantitative analysis of the data were evaluated by the aid of CFX manager software (v. 1.1.308.111; Bio-Rad, USA) (26, 27).

3.8. Primer Design

Primer sequences were designed for *TNF*, *CD40LG*, *TNFSF10*, *TNFSF8*, CD40, *TNFRSF10A*, *TNFRSF10B*, *TNFRSF11B*, *TNFRSF1A*, *TNFRSF21*, *TNFRSF25*, and *TNFRSF9* and also for the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase. For animal experiments, the primer sequences included TNF, TNFRSF10A, and β-actin. Primer Express v3.0 software was employed to design primers, which their sequences are represented in **Table 1**.

3.9. Statistical Analyses

The analyses were executed at least in triplicate, and the results were expressed as mean ± standard deviation. Differences between groups using the student's *t*-test were evaluated in SPSS 21.0 software. Values of probability (*P)* were statistically significant, if less than 0.05.

4. Results

4.1. Functionalization of SWCNTs and MWCNTs by Carboxylic Functional Group

SWCNTs and MWCNTs functionalization were

Table 1. Sequences of primers used for real-time PCR

Gene name	Sequence (5'-3')
TNF (Human)	F: GTGCTTGTTCCTCAGCCTCT R: CACCCTTCTCCAGCTGGAAG
CD40LG (Human)	F: AAACCTTGCGGGCAACAATC R: AGGCCATAGGAACCCAGAGT
TNFSF10 (Human)	F; GAGTAGAGCAGCCACAACCA R; AGTAGCTGGGACTACAGGCA
TNFSF8 (Human)	F; AAAGAATGGACCCAGGGCTG R; TGTCACCAGGGCCTGTTTTT
CD40 (Human)	F; CTGTCCATCCAGAACCACCC R; GGCAAACAGGATCCCGAAGA
TNFRSF9 (Human)	F;GCTTTTGTGCCTGTTGGGAG R; GGTACGATCTCGGCTCACTG
TNFRSF10A (Human)	F; GCATGTCAGTGCAAACCAGG R; CAAAGGGCACGATGTTTGCA
TNFRSF10B (Human)	F; CCCACCTCAGCCATCCAAAT R; TGTCCCAGCCTGTCCATAGA
TNFRSF11B (Human)	F; CCTGGCACCAAAGTAAACGC R; CTCATCCATGGGATCTCGCC
TNFRSF1A (Human)	F; CTCTCCCCTCCTCTCTGCTT R; CTGAGGCAGTGTCTGAGGTG
TNFRSF21 (Human)	F; AGGCAGGGCTGAAGAAATCC R; GAGCCGCTGGATGTAGAGTC
TNFRSF25 (Human)	F; CACTACCTGAAGGCCCCTTG R; GGCGGATCCAGATTGCTCTT
β-Actin (Human)	F; GGCACCCAGCACAATGAAG R; CCGATCCACACGGAGTACTTG
GAPDH (Human)	F; AATCCCATCACCATCTTCCA R; AAATGAGCCCCAGCCTTC
TNF (Rat)	F: CACAGAAAGCATGATCCGCG R: ACTGATGAGAGGGAGGCCAT
TNFRSF10A (Rat)	F: CACTCTCGATCCCTGATGGC R: AGGACGAAGATGAGAGCCCT
B-actin (Rat)	F: CTGGTCGTACCACAGGCATT R: TGCTAGGAGCCAGAGCAGTA

confirmed by FTIR spectroscopy. In the FTIR spectra of acid-treated CNTs, two peaks at the frequencies of ~1637 and ~1280 cm^{-1} can be related to the stretching vibrations (C=O and C–O) of the carboxyl groups (27). Other bands with the frequency of about 1033 cm^{-1} corresponded to the bending vibration (O–H) of the carboxyl groups. A broad band at the frequency of ~ 3400 cm^{-1} was related to the stretching vibration (O–H) of carboxyl group. The geometry of SWCNTs- and MWCNTs-COOH was checked by TEM, and their lengths were about 1–10 μm. In addition, their diameters and zeta potentials were 10 and 20 nm and ~-38 and ~-40 mV, respectively (27).

4.2. The Impact of Carboxylated CNTs on Cell Viability

The viability of Jurkat cells 72 h after exposure to 100 $\mu g.mL^{-1}$ of SWCNTs- and MWCNTs-COOH were 95% and 85%, respectively (27). Results indicated that MWCNTs-COOH slightly diminished the jurkat cell viability. MTT results also showed that 100 $\mu g.mL^{-1}$of acid-treated SWCNTs- and MWCNTs-COOH had no major toxicity on the cultured Jurkat cells **(Fig. 1**).

4.3. TNF Superfamily Gene Expression in SWCNTs-COOH and MWCNTs-COOH -treated Jurkat Cells

The expression of TNF family molecules altered in SWCNT-COOH and MWCNT-COOH-exposed Jurkat cells, but not in the control cells. TNF expression showed a meaningfully elevation in MWCNT-treated cells by a fold change of 5.37 ± 0.41 compared to the SWCNT-treated cells (6.38 ± 0.42; p = 0.021) and control cells (1.03 ± 0.14; p = 0.031). Expression levels of *TNFRSF10A* were also increased following exposure to CNTs. Expression fold changes of *TNFRSF10A* in SWCNTs- and MWCNTs-COOH-treated cells were 6.18 ± 0.26 and 5.7 ± 0.17, respectively compared to 1.02 ± 0.07 in the control cells (p = 0.031). No changes were observed in the expression of CD40LG in MWCNT- (1.62 ± 0.27) and SWCNT-treated Jurkat cells (1.75 ± 0.08) in comparison to the control cells (1.37 ± 0.31; p = 0.241). Although there were some changes in the expression of TNFSF10, its expression levels in SWCNTs- and MWCNTs-COOH-treated cells remained unchanged relative to the control group (-8.56 ± 2.1, -7.87 ± 1.7, and -8.17 ± 1.4, respectively; p = 0.092). There was an enhancement in the TNFRSF10B expression level in SWCNT-treated cells (4.64 ± 0.41), but expression in MWCNT-treated cells (3.37 ± 0.12) was similar to that of the control cells (3.55 ± 0.32). These expression differences did not reach the statistical significance. TNFSF8 expression in MWCNT-treated (0.09 ± 0.16), SWCNT-treated (1.69 ± 0.21), and PBS-treated cells (1.43 ± 0.09) were the same (p > 0.05). Expression of CD40 reduced in MWCNT-treated (-1.01 ± 0.08) and PBS-treated (-2.03 ± 0.28) cells but increased in SWCNT-treated cells (1.75 ± 0.11; p = 0.083). TNFRSF21 and TNFRSF9 displayed similar expression patterns. There were some elevations in the expression levels of these two genes, but no significant differences were detected between the groups (TNFRSF21 [p = 0.128] and TNFRSF9 [p = 0.452]). The expression fold changes for TNFRSF25 in MWCNT- and SWCNT-treated cells were 1.87 ±

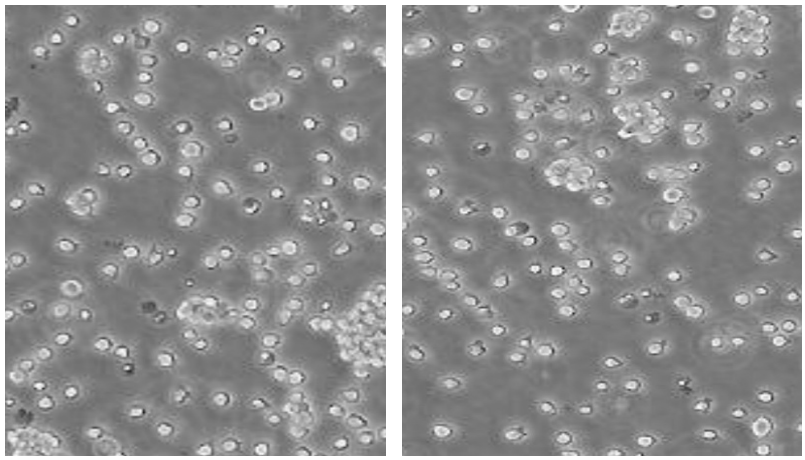

Figure 1. Jurkat cells (a) untreated and (b) treated after 24 hours using MTT assay (scale bar=100 µm)

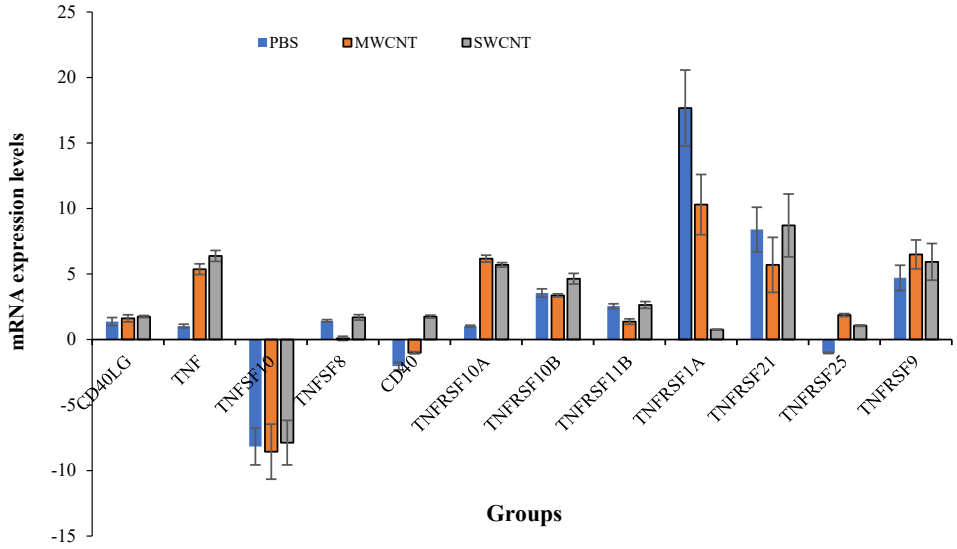

Figure 2. Relative mRNA levels of TNF family genes in the MWCNT- and SWCNT-exposed Jurkat cell line.

0.11 and 1.87 ± 0.11, respectively, and for the control cells were -1.03 ± 0.04 in ($p > 0.05$). The expression levels of TNFRSF11B and TNFRSF1A did not differ meaningfully between the groups ($p = 0.091$ and $p = 0.128$, respectively). These data are summarized in **Figure 2**.

4.4. Expression of TNF and TNFRSF10A in CNT-Treated Rat PBMCs

The expression levels of *TNF* and *TNFRSF10A* were evaluated 30 and 60 days after treatment with MWCNT, SWCNT, or PBS. After 30 days, the expression fold changes of TNF in MWCNT- and SWCNT-treated rats were 1.59 ± 0.74 and 2.3 ± 0.97, respectively. In PBS-treated rats, the expression level was 1.0 ± 0.54, indicating no significant changes ($p = 0.418$). The expression of *TNFRSF10A* was similar in all the groups (MWCNT = 4.18 ± 2.2, SWCNT = 2.70 ± 1.36, and PBS = 1.0 ± 0.39; $p = 0.348$) after 30 days. In PBMCs of CNT-treated rats, no gene expression changes were observed 60 days after treatment with CNTs. TNF expression levels in the PBMCs of MWCNT- and SWCNT-treated rat were 1.33 ± 0.03 and 1.28 ± 0.30, respectively, which was relatively similar to the expression level of PBMCs from PBS-treated rats (1.0 ± 0.43; $p = 0.290$). Thus, treatment with CNTs after the evaluation of TNF family gene expression showed no changes in the expression of this family after 60 days. Expression of *TNFRSF10A* was similar to that of TNF. Levels of *TNFRSF10A* mRNA in the PBMCs of MWCNT- (3.12 ± 1.3) and SWCNT-treated (1.42 ± 0.3) rat were the same as those of PBMCs from PBS-treated rat (1.0 ± 0.42; $p = 0.531$). All data on the expression analysis following treatment of rats with MWCNT and

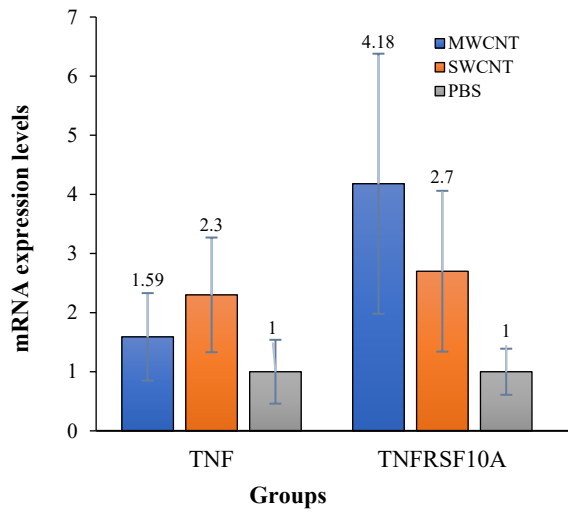

Figure 3. Relative mRNA levels of *TNF* and *TNFRSF10A* molecules in the MWCNT and SWCNT-exposed rats after 30 days.

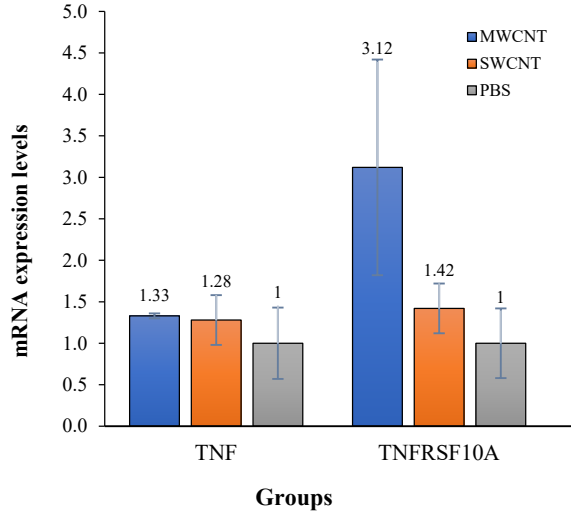

Figure 4. Relative mRNA levels of *TNF* and *TNFRSF10A* molecules in the MWCNT and SWCNT-exposed rats after 60 daysa

SWCNT after 30 and 60 days are shown in **Figures 3 and 4**.

5. Discussion

The MWCNT is carcinogenic in intraperitoneal injection, in bone marrow cells and the abdominal cavity (28-32), as well as in the lungs of mice received intratracheal instillation (33). It gives a rise to mesothelioma in tumor-prone $P53^{+/-}$ mice and in Fisher rats (30-32). Today, using CNTs as the carriers of drugs or enzymes in medical sciences is a reason for the need to investigate the toxicity of these substances (34). Jurket cell line is typically applied to study cellular signaling (35). In the present study, this cell line was selected to study the cellular signaling, including the apoptotic pathway, in terms of gene expression. Toxicity of different substances on the apoptotic pathway in Jurket cell line has already been investigated (36). The expression of TNF family genes, key players in apoptotic pathways in Jurkat cells, was analyzed, following exposure to two CNTs.

Cell viability was assessed 72 h after treatment with SWCNTs/MWCNTs, and treatment with carboxylated MWCNT alone slightly diminished the cell viability. MTT test results showed that 100 µg. mL^{-1} of acid-treated SWCNTs- and MWCNTs-COOH had no major toxicity on cultured cells. Dose-dependent toxicity of these two types of CNTs has been proven earlier (37). In thisstudy, the non-toxicity of the dose used for the induction of apoptosis was investigated.

In Lee *et al.*'s (38) study, the gills showed more sensitivity to the toxicity of MWCNT than other organs, and male fish showed higher apoptosis gene induction than females. In our studies of Jurkat cells, *TNF* and *TNFRSF10A* expression levels changed dramatically, but this difference in gene expression did not have any effect on MTT assay results. After treatment of rats with CNTs, no expression changes were observed in the mentioned genes. A possible explanation for these discordant findings is that changes in expression levels are compensated through hemostasis mechanisms. In experimental animals, exposure to MWCNTs by aspiration, inhalation, or intratracheal instillation (39-42) causes bronchiolar and alveolar hypertrophy, pulmonary inflammation, granuloma formation, and interstitial fibrosis (43-45). No data are hitherto available on human cancer owing to exposure to MWCNTs. Additionally there is no information on other MWCNT types. A number of studies have reported tumor formation in animals exposed to high doses of one type of long, straight MWCNTs, Mitsui-7. These results are inconsistent with our findings (45). Although investigations have evidenced that MWCNTs are carcinogenic, its main mechanisms are unknown and needs further systematic research (46). According to some studies, MWCNTs with larger diameters show higher toxicity against cells or in *in vivo* models (47), but the converse effect has also been described (48).

6. Conclusions

The results of 72-h cell viability assay (MTT test) in Jurkat cell line showed that carboxylic forms of SWCNT and MWCNT at the concentration of 100 µg ml^{-} had no significant effect on the cell viability. The results of cell

culture and qRT-PCR array at the cell culture indicated that the expression of two genes from TNF family genes (TNF and TNFRSF10A) increased significantly in CNT-treated cells. Examining the expression profiles of these two genes in rats revealed no significant change in the expression of any of these genes after 30 and 60 days. In order to evaluate the toxicity of carboxylated carbon nanotubes at the concentration used in this study, it is necessary to study the expression of genes of other gene families involved in the apoptotic pathway.

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