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Original Article

Carbon nanotubes induce cytotoxicity and apoptosis through increasing protein levels of Bax and ROS in mouse skin fibroblasts

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Abstract

Background and purpose: Carbon nanotubes (CNTs) are a significant discovery in nanotechnology, with widespread applications in modern technology. However, there are concerns about their potential toxicity, particularly in skin cells. This study aimed to investigate the mechanisms by which CNTs induced cytotoxicity and apoptosis in mouse skin fibroblasts.
Experimental approach: The mice skin fibroblasts were isolated and exposed to two types of CNTs at various concentrations and then analyzed for changes in viability, reactive oxygen species (ROS) production, the levels of Bcl-2-associated X protein (Bax), and lactate production.
Findings/Results: The results demonstrated that CNTs reduced cell viability and increased ROS production in a dose-dependent manner. Additionally, the current study found that CNTs increased the protein levels of Bax, a pro-apoptotic protein, in mouse skin fibroblasts. Furthermore, it was observed a significant decrease in lactate production in cells exposed to CNTs.
Conclusion and implications: The findings concluded that CNTs have the potential to be toxic substances for skin fibroblasts, which serve as the body's first line of defense. This is evidenced by their ability to increase the production of ROS and the protein levels of Bax, as well as reduce lactic acid levels. As lactic acid has been reported to have beneficial effects on skin collagen production, further studies are needed to fully understand the impact of carbon nanotube exposure on human skin health.

Keywords: Carbon nanotubes; Cell viability; Cytotoxicity; Lactate secretion; ROS generation; Skin fibroblasts.

INTRODUCTION

Nanomaterial engineering refers to the design and construction of materials, whose at least one of the dimensions has a size of 100 nm or less. Such materials exhibit unique physical, chemical, and biological properties that make them ideal for commercial and natural applications (1, 2). It has been reported that when the particles of a particular substance become smaller in the range of a few nanometers, they exhibit different characteristics such as large surface area, distinct solubility, and high mobility compared to primary particles (3).

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One of the remarkable discoveries of nanotechnology is carbon nanotubes (CNTs) (4) which have become one of the most extensively studied nanoparticles (5). Since their discovery in 1991, CNTs have shown great potential in various applications in medical sciences and modern technology, owing to their unique physical and chemical properties (6). Single-walled CNTs (SWCNT) and multiple-walled CNTs (MWCNT) are two groups of CNTs that are completely insoluble in water (7). CNTs are widely used to create optical super-media, optical devices, sensors, and catalysts due to their excellent chemical stability, unique tensile strength, as well as electrical, magnetic, thermal, and mechanical properties (8). CNTs are also used in cancer treatment and as drug carriers (9). The primary challenge and current limitation in the use of nanomaterials are related to their potential long-term toxicity, as they can pass through the body's physiological barriers due to their incredibly small size (10).

The different routes of exposure include inhalation, ingestion, and skin absorption each of which can lead to unique toxicity outcomes. In particular, the skin absorption of carbon nanoparticles is an important issue that requires further investigation (11). Skin is one of the body's major organs and is of great interest due to its functional and restorative properties (12). One of the skin's primary functions is to act as a barrier for protecting the body against the entry of external factors and preventing dehydration (13). Fibroblast cells are the essential components of connective tissue and can be found throughout the body, with different functional roles based on their location (14). In the skin, dermal fibroblasts organize and make up the connective tissue. These cells are responsible for cell synthesis and the secretion of connective tissues including the different types of collagens, elastin fibers, and precursor molecules. Fibroblasts are spindle-shaped and possess all the characteristics of active protein-producing cells (15).

The interaction between nanoparticles and skin is still under investigation as nanoparticles with a size less than 4 nm have been shown to penetrate healthy skin (16). The main mechanism by which nanoparticles function is not yet known, but *in vivo* and *in vitro* experiments showed that nanoparticles are capable of producing reactive oxygen species (ROS) (17). ROS can lead to significant toxicity, including the induction of oxidative stress, apoptosis, and the inhibition of cell proliferation (18). Additionally, exposure to nanoparticles has been observed to increase lung inflammation and oxidative stress in animals (19).

Given the increased production and the use of these nanoparticles, which have spread in large quantities in the environment and posed potential risks to human health and the environment, the current study aimed to investigate the effect of CNTs on the viability of skin fibroblast and determine possible mechanisms. Furthermore, to gain new insight and a better understanding of the mechanisms involved in carbon nanotube toxicity, this study evaluated the Bcl-2-associated X protein (Bax), a member of the Bcl-2 family known to play a role in apoptosis, in skin fibroblast treated with CNTs.

MATERIALS AND METHODS

Agents

The agents used in this study included the rabbit anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Cat. No. 181603, Abcam, USA), rabbit anti-Bax (Cat. No. ab32503, Abcam, USA), fetal bovine serum (FBS), penicillin/streptomycin (Gibco, USA), 2, 7-dichlorofluorescein diacetate (DCFH-DA) (CAS No. 4091-99-0, sigma, USA), the lactate assay kit (Pars Azmoon, Iran), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra zolium bromide (MTT) (CAS No. 298-93-1, Sigma, USA), low glucose Dulbecco´s Modified Eagle Medium (DMEM), 0.25% trypsin-EDTA (Bio-Idea, Iran).

Carbon nanotubes

This study used two types of CNTs including SWCNT and MWCNT. The nanotubes were generously gifted by Dr. Zinali from the Research Institute of Petroleum Industry, Tehran, Iran (> 95% purity by weight, outer diameter of 8-15 nm, inner diameter of 3-5 nm, 50 µm length) (20).

Isolation and primary culture of skin fibroblasts

In this experimental study, the fibroblast cells were isolated from newborn C57BL/6 mice purchased from the animal house of Ahvaz Jundishapur University of Medical Sciences (AJUMS). Animal procedures were approved by the Ethics Committee of the Care and Use of Laboratory Animal Resources of AJUMS for the control and supervision of experiments on animals (Ethics code: IR.AJUMS.REC.1395.401).

The skin fibroblasts were isolated and cultured in the following steps: the isolated skin was minced into smaller pieces. Then, the pieces were washed twice with Dulbecco´s phosphate-buffered saline (DPBS), and incubated with 2 mL of collagenase in a shaker incubator at 37 °C for 20 min. After aspirating the DPBS, the collagenase was neutralized with DMEM/10% FBS and replaced with 2 mL of 0.25% trypsin diluted with sterile DPBS (1:1), and then incubated in the shaker incubator at 37 °C for 30 min. To obtain single cells, the samples were gently pipetted up and down. Next, trypsin was neutralized with DMEM/10% FBS. Finally, the cell pellet was obtained by centrifugation at 1000 rpm for 3 min. After that, the fibroblasts were cultured in a DMEM low glucose medium containing 10% FBS and 1% penicillin/streptomycin (pen/strep) at 37 °C and 5% CO_2. The mice skin fibroblasts were sub-cultured in 12-well plates or 7 cm dishes containing low glucose DMEM, 10% FBS, and 1% pen/strep for the designed experiments.

Exposure of mouse skin fibroblasts to carbon nanotubes

The cells were seeded at a density of 5×10^4 cells/well in a 12-well culture plate and exposed to SWCNT (concentrations of 0, 1, 2, 5, 10, 15, 20, 50, 75, 100, 150 and 200 ng/mL) and MWCNT (concentrations of 0, 1, 2, 5, 10, 15, 25, 50, 75, 100, 150 and 200 ng/mL) for 24 h for the further analysis.

MTT assay

The effect of CNTs on cell viability was determined using the MTT assay. Briefly, cells were seeded at a density of 5×10^4 cells/well in a 12-well culture plate, and exposed to the different concentrations of CNTs at 37 °C for 24 h. After the incubation time, the culture medium was discarded from each well, and the cells were washed twice with phosphate-buffered saline (PBS), then 500 µL of MTT assay solution was added to each well and incubated at 37 °C for 4 h. After that, the formazan crystals were dissolved by adding 1000 µL of dimethyl sulfoxide to each well, followed by incubation at 37 °C for 20 min. Finally, the absorbance was measured at 570 nm using a spectrophotometer.

Measurement of reactive oxygen species

The intracellular generation of ROS was measured using DCFH-DA, which is an oxidation-sensitive fluorescence probe. In summary, skin fibroblasts were seeded into a 12-well culture plate. After treatment with the different concentrations of CNTs for 24 h, the cells were washed twice with PBS and loaded with 25 µM of DCFH-DA, then incubated at 37 °C for 20 min. At the end of the incubation period, DCF fluorescence was measured at the excitation wavelength of 488 nm and the emission wavelength of 521 nm.

Measurement of lactate production

The release of lactate was measured by using a lactate kit according to the manufacturer's instructions. Briefly, cells were seeded into a 12-well culture plate at a density of 5×10^4 cells/well. After 24 h, the cells were treated with different concentrations of CNTs. After 24 h of exposure to CNTs, the released lactate in the medium was measured using the lactate kit.

Western blotting analysis

Mouse skin fibroblasts were treated with CNTs for 24 h, and the expression of Bax was determined using rabbit anti-Bax antibody. Briefly, cells were seeded at a density of 3×10^6 cells/well and treated with CNTs. After 24 h of exposure to CNTs, the cells were harvested and lysed using radioimmunoprecipitation assay buffer. The total protein concentration was determined using the Lowry protein assay, and equal amounts of protein were separated by 10% sodium dodecyl

sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After blocking with 5% non-fat milk for 1 h, the membranes were incubated with the primary antibody of Bax for 5 h at 4 °C. Subsequently, the membranes were washed three times with Tris-buffered saline with 0.1% tween (TBST) and exposed to the secondary antibodies (anti-rabbit antibody Cat. No. ab.065M4769V, Sigma, USA) for 1 h at room temperature. After washing with Tris-buffered saline and TBST, the bands related to Bax were detected using the enhanced chemiluminescence western blotting system. Equal protein loading was verified by probing with the anti-GAPDH antibody.

Statistical analysis

All data were expressed as mean ± SD and analyzed by Prism software. The data were analyzed by one-way ANOVA followed by Tukey post-hoc test. In addition, the analysis of an independent Student T-test was used to compare the protein level of Bax between the control group and the SWCNT or MWCNT group. ImageJ software was used to quantify protein band densities. A value of $P < 0.05$ was considered statistical significance.

RESULTS

Cell viability in response to carbon nanotubes exposure

The viability of skin fibroblasts was measured in the presence of CNTs at different concentrations by using the MTT reduction method, and the results were shown in Fig.1. Skin fibroblasts were exposed to SWCNT (Fig. 1A) and MWCNT (Fig. 1B) at the different concentrations of 0 (control) to 200 ng/mL (as shown in Fig.1) for 24 h. The results showed that cell viability decreased significantly in a dose-dependent manner after exposure to CNTs (Fig. 1).

Generation of intracellular reactive oxygen species due to carbon nanotubes exposure

The high level of ROS in cells causes cell damage and apoptosis (21). In this study, ROS generation was determined with DCFH-DA. The results showed that exposure to CNTs caused a significant increase in the level of ROS compared to the control group (Fig. 2).

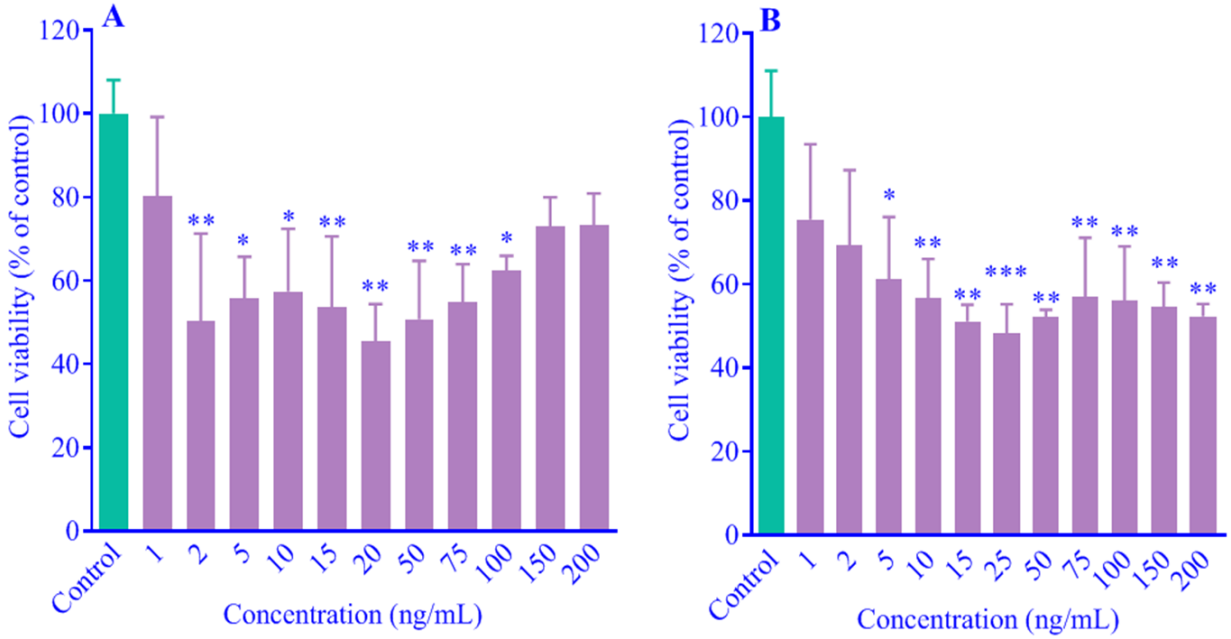

Fig. 1. Cell viability of mouse skin fibroblasts following the treatment with (A) SWCNT and (B) MWCNT by MTT assay. Data were shown as mean ± SD of the triplicate samples. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ represent the significant differences from the control. SWCNT, Single-walled carbon nanotubes; MWCNT, multiple-walled carbon nanotubes; MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

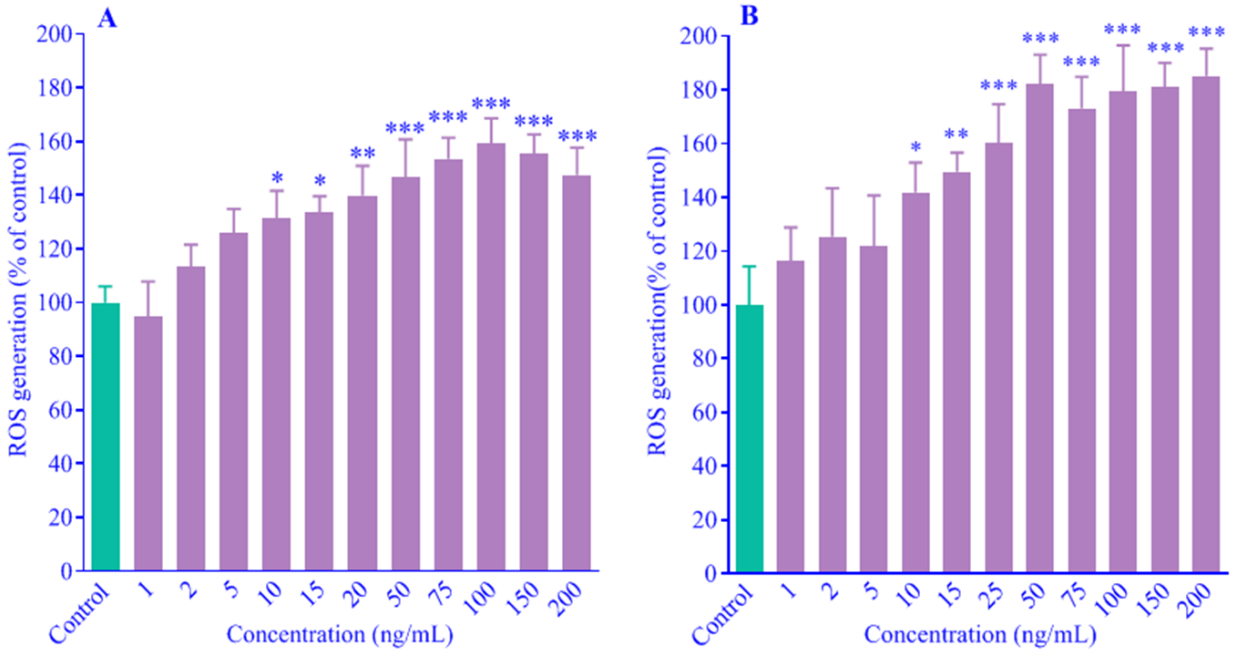

Fig. 2. ROS generation in mouse skin fibroblasts following the treatment with (A) SWCNT and (B) MWCNT. Data were represented as mean ± SD of the triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ represent significant differences than control. ROS, Reactive oxygen species; SWCNT, Single-walled carbon nanotubes; MWCNT, multiple-walled carbon nanotubes.

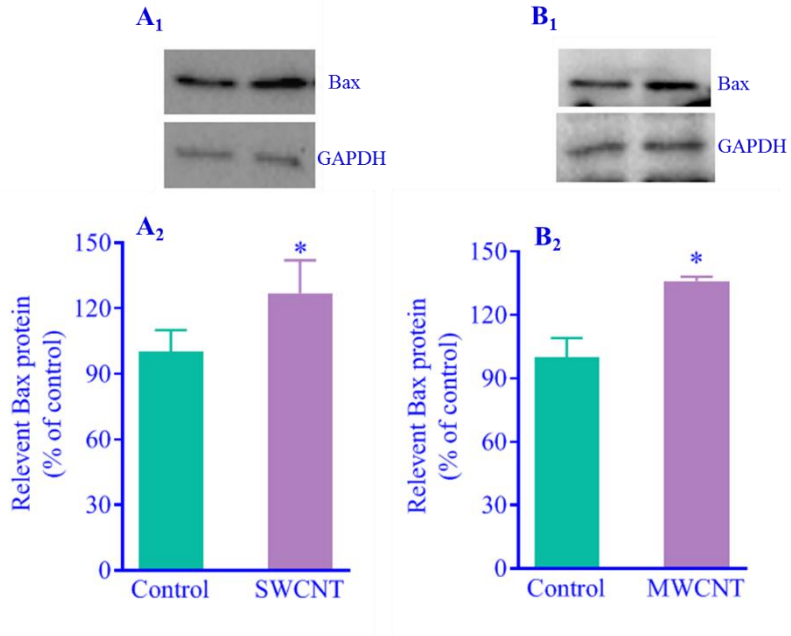

Fig. 3. Protein levels of Bax in mouse skin fibroblasts following the treatment with (A) SWCNT and (B) MWCNT using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses. Data were represented as mean ± SD of the triplicate samples. $^{*}P < 0.05$ represents a significant difference from the control. Bax, Bcl-2-associated X; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SWCNT, single-walled carbon nanotubes; MWCNT, multiple-walled carbon nanotubes.

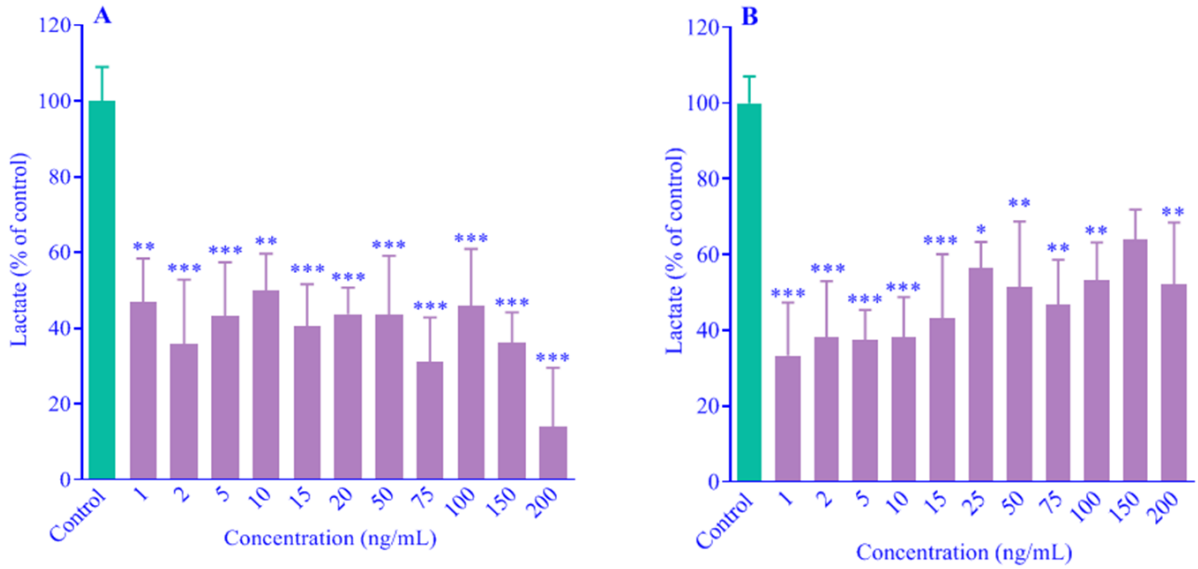

Fig. 4. Lactate production in mouse skin fibroblasts following the treatment with (A) SWCNT and (B) MWCNT measured by lactate kit. Data were represented as mean ± SD of the triplicate samples. *P < 0.05, **P < 0.01, and ***P < 0.001 represent significant differences from the control. SWCNT, Single-walled carbon nanotubes; MWCNT, multiple-walled carbon nanotubes.

Effect of carbon nanotubes on the protein levels of Bax by western blotting

To investigate the effect of CNTs on apoptosis, mouse skin fibroblasts were isolated and treated with 10 ng/mL of SWCNT or MWCNT and then incubated for 24 h. The protein levels of Bax, an apoptotic marker, were visualized using the specific antibody against the Bax. Figure 3 demonstrates that the protein levels of Bax were significantly increased when skin fibroblasts were exposed to CNTs compared to control, indicating a possibility that the apoptotic pathway can be induced in skin fibroblasts by CNTs.

Effect of carbon nanotubes on lactate secretion

Healthy fibroblasts secrete lactate as a signaling component to increase fibroblast proliferation and collagen synthesis to maintain tissue growth, maintenance, and repair (22). There was a decrease in lactate secretion in the medium when the cells were exposed to both SWCNT (Fig. 4A) and MWCNT (Fig. 4B).

DISCUSSION

CNTs have potential applications in medicine and other fields, but there is a significant gap in current knowledge regarding their impact on human health and the environment. It has been documented that CNTs are toxic to cells and have the most significant impact on the skin, which is the body's first line of defense (23). Moreover, CNTs can translocate to the circulatory system and cause damage to cells and tissues throughout the body (24)

The current study findings indicated that the exposure of skin fibroblasts to CNTs induces cytotoxicity, as evidenced by a decrease in cell viability, lactate release, an increase in the intracellular generation of ROS, and a more significant increase in the protein level of Bax as a pro-apoptotic agent.

Numerous studies have shown that CNTs are toxic and induce cell death (25-27). MTT assay results in this study demonstrated that CNTs in the concentrations used (1-200 ng/mL) are toxic to skin fibroblasts. Our findings were consistent with a report demonstrating that CNTs induce cytotoxicity in human lung cells (28). Also, some studies have investigated the effects of MWCNT on allergic airway inflammation in mice and shown that exposure to MWCNT can exacerbate the inflammation in the animals, potentially through the activation of inflammasome pathways (29) Cavallo *et al.* demonstrated that the concentrations of CNTs greater than 10 µg/mL produce cytotoxicity in

human lung epithelial cells (30), while the present study found that CNTs induced cytotoxicity in skin fibroblasts at low concentrations (nanogram values) (Fig.1). These results suggest that skin fibroblasts are likely more sensitive to CNTs compared to lung cells.

Reduction in cell viability prompted us to investigate the factors participating in the decrement of cell viability such as ROS production. The generation of ROS as a well-known factor can lead to apoptosis (31) and maybe a reason for the reduction in cell viability observed in the current study. CNTs can induce oxidative stress (32) upon entering cells, which can increase cell damage through mitochondrial dysfunction and an oxidant/anti-oxidant imbalance. The present observations showed a significant increase in ROS production in skin fibroblasts treated with either SWCNT or MWCNT, indicating that ROS generation may be one of the causes of cell damage in CNTs-induced toxicity and subsequent reduction in cell viability (33).

Literature has shown that ROS can affect apoptosis-related proteins in various cell types (34). To explore this possibility in the current study, the protein levels of Bax were analyzed in skin fibroblasts in response to carbon nanotube exposure. Bax is an important pro-apoptotic regulatory protein in the Bcl-2 family that can activate the apoptotic pathway (35). Western blot analysis (Fig. 3) revealed an increase in Bax protein levels after carbon nanotube treatment in mouse skin fibroblasts. Thus, it seems that CNTs may trigger ROS generation, followed by an increase in the protein level of Bax, ultimately leading to the activation of the apoptosis pathway and reduction in cell viability in skin fibroblasts.

In normal conditions, fibroblasts play a crucial role in maintaining skin morphology and health, as well as preventing disease through the synthesis and secretion of collagen, fibronectin, and growth factors (36). Additionally, fibroblasts synthesize and secrete lactate as a signaling molecule to communicate with neighboring cells and increase collagen synthesis, thus promoting tissue growth, maintenance, and repair (22). There is a positive correlation between lactate production and collagen secretion, indicating lactate secretion levels could serve as a good indicator of cell health or damage following carbon nanotube exposure (37,38).

This study found that the exposure of mouse skin fibroblasts to CNTs led to a significant decrease in lactate production (Fig.4). Given the direct correlation between lactate levels and collagen secretion, it is thought that CNTs may have an impact on the production and the release of collagen in skin fibroblasts. These findings suggest that exposure to CNTs may hurt the overall health and the function of skin fibroblasts. This issue highlights a need for further research to better understand the effects of CNTs on human health and the environment.

CONCLUSION

In conclusion, the present study has demonstrated that CNTs can be cytotoxic to skin fibroblasts even at low doses. This cytotoxicity may be attributed to the activation of the Bax apoptotic pathway, which may result from the increased production of ROS. Additionally, since lactic acid is known to have a positive impact on skin collagen synthesis, the suppression of lactate generation by CNTs may lead to reduced collagen production, ultimately accelerating the aging process in the skin. However, further research is necessary to comprehensively understand the effects of carbon nanotube exposure on human skin health.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

A. Kheirollah designed the study revised and approved the manuscript. Z. Nazeri and S. Azizidoost performed the laboratory tests and prepared the manuscript. M. Jamalan, M. Cheraghzadeh, and V. Zarezade analyzed the data and revised the manuscript.

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