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Original Article

Assessment of the Skin and Heart Tissue Damage Following Inhalation of Carbon Nanotubes in Wistar Rats Using Isolated Mitochondria

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Abstract

The unique properties of carbon nanotubes (CNTs) have led to their use in various fields. But, the toxicity of CNTs has been reported in biological and environmental systems. The aim of this research is to study the effect of multi-wall carbon nanotubes (MWCNTs) through inhalation chamber on the mitochondrial damage and oxidative stress using the mitochondria obtained from the skin and heart. Rats were exposed to 5 mg/m^3 of MWCNTs (10 nm) aerosol for 5 hours /day, 5 days/week for 2 weeks in a whole-body exposure chamber. After 2-weeks exposure, Heart and skin mitochondria were evaluated for evaluation of toxicity parameters. The results showed that nanoparticles significantly decreased mitochondrial succinate dehydrogenase (SDH) activity and increased the level of reactive oxygen species (ROS), collapse in mitochondria membrane potential (MMP), swelling in mitochondria, and cytochrome release. In conclusion, we suggested that 5 mg/m^3 of MWCNTs (10 nm) induce ROS mediated cytotoxicity by directly targeting mitochondria in both skin and heart tissue.

Keywords: Heart, Mitochondria, Multi Wall Carbon Nanotube, Skin, Toxicity.

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1. Introduction

The unique properties of carbon nanotubes (CNTs) have led to their use in various fields Including industry, agriculture and medicine. But, the toxicity of CNTs has been reported in biological and environmental systems [1-4]. Statistics show that hundreds of tons of nanoparticles (NPs) enter the environment annually, but there is not much evidence about

the adverse effects of nanomaterial on biological systems [5]. NPs may enter the body through various routes such as inhalation, injection, dermal penetration and ingestion, and may be distributed to various tissues via the circulatory system [6]. Human exposure to CNTs is primarily through inhalation and dermal contact. Skin is a tissue that is directly exposed to CNTs and the heart is important as a tissue that is involved in indirect and secondary exposure to CNTs [7]. Several studies show that carbon nanotubes (CNTs) are toxic for organisms (especially for humans), and presence of CNTs in the environment modify the physicochemical behavior the several of environmental pollutants [8].

There are two forms of CNTs that are single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) [9-11]. *In vivo* and *in vitro* studies show that NPs have the potential to induce an oxidative stress process, and is recognized as one of the most important mechanisms of toxicity by NPs [12, 13]. Oxidative stress is known as an imbalance between the generation of free radicals and the antioxidant defense system. Furthermore, oxidative stress has been illustrated in a variety of target cells resulting exposure to CNTs [14]. One of the most important consequences of oxidative stress is the generation of reactive oxygen species (ROS), as well as damage to cellular components [4]. Research shows that ROS play a role in many physiological processes, including cell growth, proliferation, and also apoptosis [15, 16].

It has been shown that CNTs (MWCNTs) are capable of generation of ROS through damage to cellular components, especially mitochondria. Furthermore, studies have shown that MWCNTs induce cell death and DNA damage via oxidative stress and ROS [12]. Mitochondria is one of the vital organelle that has unique functions. This organelle plays a role in the generation of ROS, cellular energy production, and also ionic regulation [17]. The aim of this research is to study the effect of MWCNT through inhalation chamber on the mitochondrial damage and oxidative stress using the mitochondria obtained from the skin and heart tissue.

2. Materials and Methods

2.1. MWCNT

For present study, we were purchased MWCNTs from Tehran Oil Research Institute. These MWCNTs (10 nm) were with 99.8% purity (wt/wt).

2.2. Animals

The animal was acquired from the Pasteur Institute. All experimentations were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.3. The Research Model

10 male rats in the chamber were exposed to either clean air (control) or MWCNTs at concentration of 5 mg/m^3 (10 nm) for 5 hours a day, 5 days a week and for 2 weeks. At the end of the 2-week exposure period, rats from

each group (control and test) were killed, and skin and heart were isolated. Then, skin and heart mitochondrial isolation were performed and parameters of mitochondrial toxicity were assessed.

2.4. Inhalation Chamber

A cross-sectional view of exposure chamber was [90-60-50 cm polycarbonate Plexiglas] and flow air [primary air flow rate of 25 l/min] and the chambers have 56 l/min enough circulation air for 5 h. This chamber was designed to operate within a standard two ventilators on the roof. Hold up to 10 rat in open mesh cages suspended above bedding material to maximize the free flow of particles around the rat and minimize crowding. In this study, we used two exposure chambers: one for exposure to different type of MWCNTs (5 mg/m^3) for 5 h/day, 5 days/week for 2 weeks (as exposed groups) and the other chamber for exposure to fresh air (as a control or unexposed group). A concentration (5 mg/m^3) of MWCNTs has been obtained using the pilot study and based on previous studies.

2.5. Mitochondrial Isolation from Rat Skin and Heart

Rats were decapitated and the heart and skin were surgically harvested, minced and homogenized with a glass hand held homogenizer with previous method. Differential centrifugation (10 min at 1500× g for the first stage and 10 min at 10 000×g for the second stage at 4℃) was used for isolation of mitochondria from heart. Furthermore, differential centrifugation (10 min at 1000× g for the first stage and 10 min at 10 000×g for the second stage at 4℃) was used for isolation of mitochondria from skin. Mitochondrial sediments were suspended in the corresponding buffer at 4℃ to assess mitochondrial toxicity parameters [18, 19].

2.6. Succinate Dehydrogenase (SDH) Activity Assay

The activity of SDH was measured by MTT (0.4% w/v) test. The mitochondrial suspension from skin and heart were incubated at 37℃ for 30 min. After that, the product of formazan crystals was dissolved in 100 ml DMSO and the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) [20].

2.7. Mitochondrial ROS Level Assay

To perform this test, isolated mitochondria from skin and heart were suspended in respiration assay buffer. Then, DCFH-DA (Fluorescent probe) used for the ROS generation from mitochondria at the λ Ex = 488 nm, and λ Em= 527 nm [21].

2.8. Mitochondrial Membrane (MMP) Assay

Briefly, the mitochondria from skin and heart were isolated and then were suspended in the MMP assay buffer. Eventually, Rhodamine 123 (Rh 123), as a mitochondrial specific fluorescent probe, used for the MMP assay at the λ Ex = 490 nm and λ Em = 535 nm [22].

2.9. Determination of Mitochondrial Swelling

In this study, isolated mitochondria from skin and heart were suspended in swelling assay buffer. Then, absorbance was measured at 540 nm using an ELISA reader (Tecan, Rainbow Thermo, Austria). The decrease in absorbance reflects increasing swelling in the mitochondria.

2.10. Cytochrome C Release Assay

The concentration of cytochrome *c* was determined by using the Quantikine Rat/Mouse Cytochrome *c* Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn.).

2.11. Statistical Analysis

Data are reported as mean ± SD. All statistical analyses were performed using Graph Pad Prism (GraphPad Prism software, version 6). The assays were performed 5 times. Statistical significance was determined using the t-test. Statistical significance was set at $P<0.05$.

3. Results and Discussion

3.1. Results

3.1.1. Mwcnts Decreased the SDH Activity

As shown in Fig. 1, MWCNTs have been able to decrease the SDH activity in the mitochondria isolated from the skin and heart tissue. Decrease in activity of SDH represents a decrease in mitochondrial function.

3.1.2. Mwcnts Increased the ROS Generation

Compared to the control group, the level of ROS generation in the mitochondria isolated from skin and heart was significantly increased in exposure to MWCNTs (Figure 2). Furthermore, an increase in fluorescence intensity (DCF) indicates an increase in ROS generation.

3.1.3. Mwcnts Increased the MMP Collapse

In this study, there was a significant increase ($P < 0.05$) in the MMP collapse after exposure to MWCNTs in the both test groups (Figure 3). An increase in fluorescence intensity (Rh123) indicates an increase in MMP collapse.

3.1.4. Mwcnts Increased the Mitochondrial Swelling

As shown in Fig. 4, MWCNTs have been able to increase the mitochondrial swelling in the mitochondria isolated from the skin and heart tissue. Decrease in absorbance represents an increase in the mitochondrial swelling.

3.1.5. Mwcnts Induced the Cytochrome C Release

In this study, there was a significant increase ($P < 0.05$) in the cytochrome c release after exposure to MWCNTs in the both test groups (Figure 5).

3.2. Discussion

In this study, we examined the toxic inhalation effect of MWCNTs on skin and heart through assessing oxidative stress, and several mitochondrial toxicity parameters. Reports have shown that CNTs are interacted with cellular membranes through unique route and also enter into mammalian cells through various pathways [12]. CNTs have been studied as a risk factor for cardiovascular [23].

In many studies, animal and cellular models have been used to evaluate the acute and chronic toxicity of CNTs. It has been shown that cellular exposure to CNTs has been associated with several physiological changes, including induction of apoptosis and an increase in the generation of ROS [2, 24]. *In vivo* and *in vitro* models have helped to better understand the mechanism of toxicity caused by the NPs. The use of the exposure chamber is one of the models that importantly helps to identify the toxicity of NPs [25-27].

Initially, the results showed that MWCNTs reduced the mitochondrial SDH activity. Oxidative stress and subsequent generation of ROS is one of the most important mechanism involved in the toxicity of NPs [28]. In this study, oxidative stress as a main mechanism of MWCNTs toxicity was determined. Oxidative stress was investigated by determining mitochondrial ROS level. The results showed that MWCNTs increased the level of ROS generation in the mitochondria isolated from the skin and heart. DCFH-DA has been very popular as fluorescent probe for ROS generation in nano-toxicology research, and it can be used to evaluate a number of oxidizing species [29]. The results are in agreement with previous studies [29].

Decline of MMP was one of the indication of cellular cell death (apoptosis), and Rh123 was a membrane potential–sensitive cationic fluorescent probe that could evaluate the level of MMP collapse. The results showed that MWCNTs increased the MMP collapse in the mitochondria isolated from the skin and heart. One of the most important mitochondrial functions is to preserve the MMP. The decline of MMP is able to decrease the permeability of the mitochondrial membrane, which not only means the decline of mitochondrial function but also the initial indicator of cell death (apoptosis) [17].

The results showed that MWCNTs increased the mitochondrial swelling, and cytochrome c release in the mitochondria isolated from the skin and heart. Studies have shown that CNTs can induce apoptosis signaling [12, 30]. Mitochondrion is one of the vital organs that plays an important role in the generation of ROS and signaling apoptosis [31-33]. Release cytochrome c from mitochondria to the cytosol is involved in the activation of signaling apoptosis [34, 35]. The results of our study showed that MWCNTs lead to cytochrome release, which ultimately can induce apoptosis in isolated mitochondria from the skin and heart. In addition, the increase of ROS level could adjust the MMP, starting the release of cytochrome *c* to the induction of the apoptotic signaling [36]. High levels of ROS, mitochondrial dysfunction, and also induction of apoptosis signaling play an important role in the pathogenesis of some diseases (such as neurodegenerative diseases) [31].

4. Conclusion

In conclusion, exposure to MWCNTs is associated with the numerous consequences, including an increase in ROS generation, MMP collapse cytochrome c release,

mitochondria swelling, and a decrease in SDH activity. In recent decades, MWCNTs are considered as the pollutant of the atmosphere. However to protect the society, good ventilation, appropriate personal protective equipment are recommended.

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Figures:

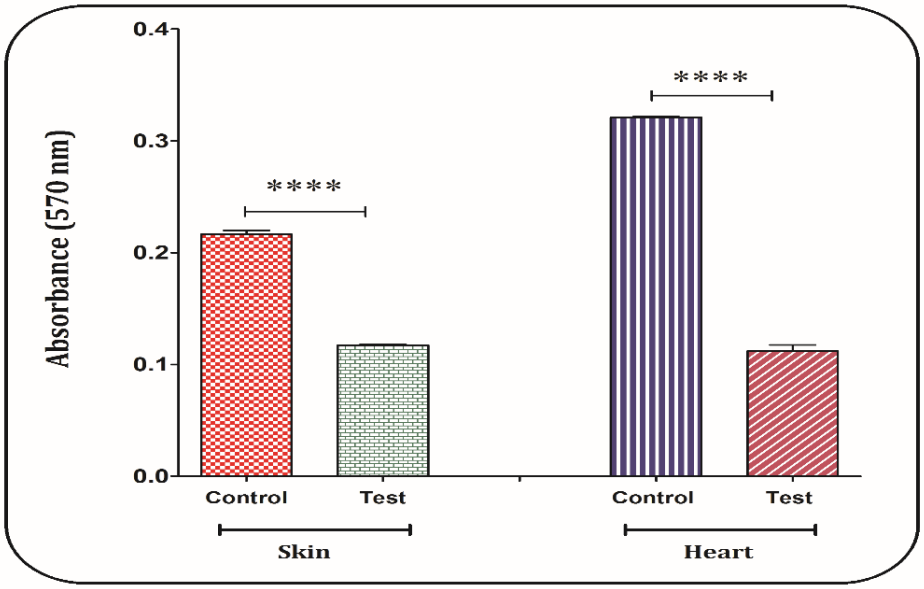

Figure 1. SDH activity assay. The effect of MWCNTs on the SDH activity in the mitochondria isolated from the Skin and Heart. Data are presented as mean ± SD (n=3). The t-test was carried out. **** shows a significant difference in comparison with the control group (P<0.0001).

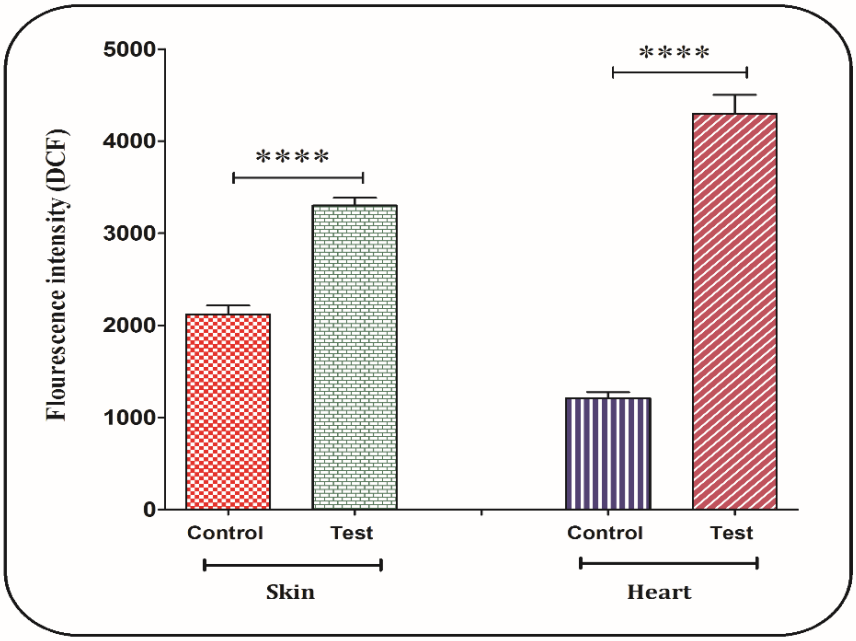

Figure 2. ROS formation assay. The effect of MWCNTs on the ROS formation in the mitochondria isolated from the Skin and Heart. Data are presented as mean ± SD (n=3). The t-test was carried out. **** shows a significant difference in comparison with the control group (P<0.0001).

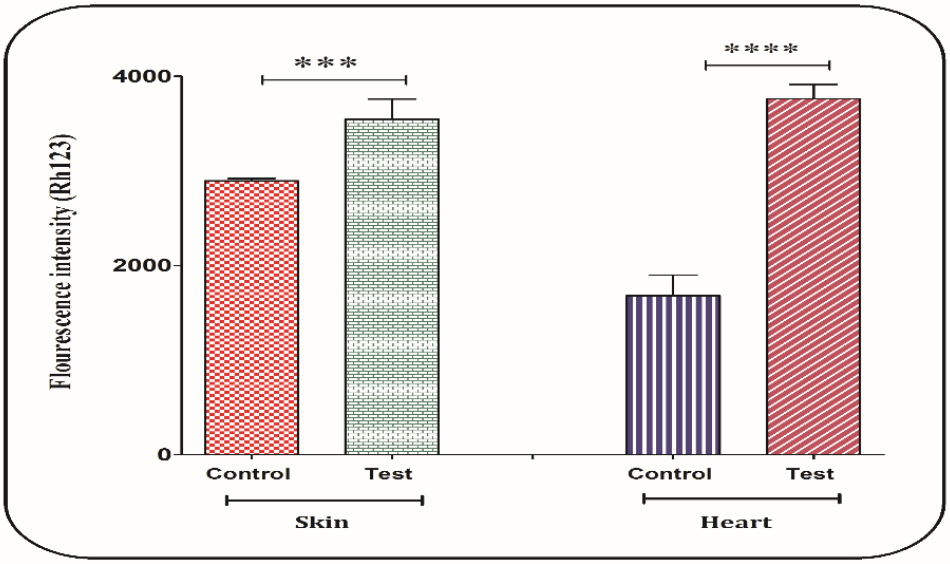

Figure 3. Mitochondrial membrane potential (MMP) assay. The effect of MWCNTs on the MMP collapse in the mitochondria isolated from the Skin and Heart. Data are presented as mean ± SD (n=3). The t-test carried out. *** and **** show a significant difference in comparison with the control group (P<0.001 and P<0.0001, respectively).

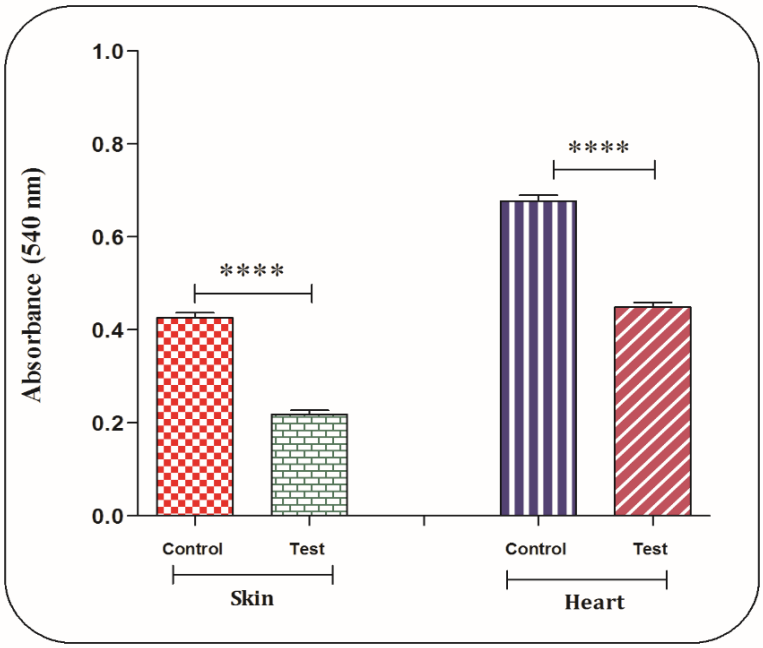

Figure 4. Mitochondrial swelling assay. The effect of MWCNTs on the Mitochondrial swelling in the mitochondria isolated from the Skin and Heart. Data are presented as mean ± SD (n=3). The t-test was carried out. **** shows a significant difference in comparison with the control group (P<0.0001).

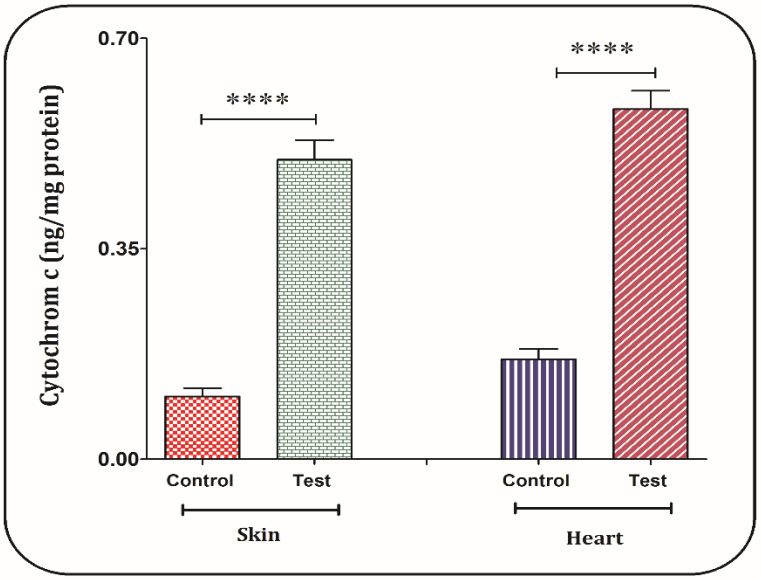

Figure 5. Cytochrome c release assay. The effect of MWCNTs on the cytochrome c release in the mitochondria isolated from the Skin and Heart. Data are presented as mean ± SD (n=3). The t-test was carried out. **** shows a significant difference in comparison with control group (P<0.0001).