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Effects of Caffeic Acid on Multi-walled Carbon Nanotubes -Induced Mitochondrial Toxicity in Rat Kidney

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1 Original submission

3 comments · 30 Dec 2022 – 5 Jan 2023

Referee 1 Reviewer

MAJOR REVISION 30 Dec 2022

1. Considering that this study aims to evaluate the ability of caffeic acid to reduce the toxicity induced in kidney tissue by carbon nanotubes, why were specific tests for measuring kidney function not used?
2. Are the protective effects claimed in this study all related to the antioxidant effects of caffeic acid, and in this case, what would be the advantage of caffeic acid over cheaper antioxidants such as vitamin C?
3. In picture number 3, the abbreviation DCF should be given in full in the subtitle.
4. In the graphs, the colors used for the columns are very similar to one another, and distinctive colors and designs should be used for each column in the graph.

Referee 2 Reviewer

MAJOR REVISION 31 Dec 2022

1- Please write the name of the city of Ahvaz correctly in all parts.
2- There is no reference for mitochondrial isolation. Provide a reference for your method.
3- In the methods section, the author mentions 320 microliters of MWCNTs but does not mention this in the results section.
4- Discuss the power of the article in more detail. What is the power of this study?

Editor-in-Chief

MAJOR REVISION 5 Jan 2023

Dear Authors,
Your manuscript needs significant changes to make it suitable for publication in JJNPP. For your reference, the reviewers' comments are attached. Please revise the manuscript accordingly and upload it with a point-by-point response to the reviewers' comments or any rebuttal.
Regards,
JJNPP EIC

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

1- *Considering that this study aims to evaluate the ability of caffeic acid to reduce the toxicity induced in kidney tissue by carbon nanotubes, why specific tests for measuring kidney function were not used.*

The purpose of this study was to investigate the protective effect of caffeic acid against the toxicity of nanotubes. In addition, due to the lack of funds and sufficient facilities, kidney function tests were not investigated.

2- *Are the protective effects claimed in this study all related to the antioxidant effects of caffeic acid, and in this case, what would be the advantage of caffeic acid over cheaper antioxidants such as vitamin C?*

Due to the need to identify new medicinal compounds in toxicology and also the availability of this substance in the diet of people, we investigated the protective effects of this substance.

3- *In picture number 3, the abbreviation DCF should be given in full in the subtitle*.

The subtitle was corrected.

4- *In the graphs, the color spectrum used to color the columns is very close to each other, and distinctive colors and designs should be used for each column in the graph.*

The graphs were modified.

5- Please write the name of the city of Ahvaz correctly in all parts.

The word Ahvaz was corrected.

6- There is no reference for mitochondrial isolation. Bring a reference for your method.

References 14 and 15 are related to this part.

7- In the method part, the author mentions 320 microliters of MWCNTs, but didn't in the result.

This part was corrected in the text

8- Discuss more about the power of the article. What is the power of this study?

The results of the study showed that the activity of antioxidant enzymes in the groups treated with CA was significantly enhanced compared to the MWCNTs group, which indicates the anti-oxidative effect of CA on mitochondria. The main finding of this research is that CA showed a protective effect against mitochondrial toxicity caused by MWCNTs.

Effects of Caffeic Acid on Multi-Walled Carbon Nanotubes -Induced Mitochondrial Toxicity in Rat Kideny

Abstract

Multi-walled carbon nanotubes (MWCNTs) are being utilized in a wide variety of fields. With regard to their numerous applications, MWCNT-induced toxicity has not been extensively investigated. Thus, the present study sheds light on the protective effect of caffeic acid (CA) on mitochondrial toxicity in the kidney caused by MWCNTs in Wistar rats employing the MTT assay as well as reactive oxygen species (ROS) indices, based on measuring glutathione (GSH), mitochondrial membrane potential (MMP), malondialdehyde (MDA), and catalase (CAT) activity. According to the MTT assay, use of MWCNTs could significantly diminish mitochondrial viability based on doses. Furthermore, the study findings suggested that MWCNTs could reduce GSH content and CAT activity, and subsequently improve mitochondrial ROS formation and damage the mitochondrial membrane of the kidney. The findings also implied that CA could protect renal mitochondria against toxicity induced by MWCNTs through lowering oxidative stress.

Keywords: Carbon Nanotubes, Caffeic Acid, Oxidative Stress, Kidney, Mitochondria

Introduction

With one main dimension less than 100 mm as a minimum, nanomaterial's are endowed with novel properties and are also characterized by their high potentials for further applications compared with their original forms (1). Despite numerous uses of such materials, few research studies have focused on their effects on humans and the environment. Accordingly, it is of utmost importance to investigate toxic effects as well as their mechanisms since they can generate harmless products (2). Among nanoparticles, carbon nanotubes (CNTs) are approving of interest for their electrical, mechanical, and magnetic properties. In this respect, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) have been recognized as two forms of nanotubes (3). However, there are few studies concerning their toxicity, whose results are often inconsistent. It has been also reported that such materials can enter the working environment as suspended solids in absorbing quantities and, possibly, cause industrial hazards (4). In addition, it has been stated that they can produce CNTs in sizes <2.5μm due to the burning of methane and propane of natural gas in ordinary stoves inside and outside houses (4). Studies on toxic effects of CNTs merely center on the toxicity of these substances on inhalation and epidermal contacts, because the greatest possible contacts with nanomaterial's are through the skin or the respiratory tract (5, 6). Studies in the related literature have revealed that CNTs can be absorbed into the circulatory system after entering the lungs and then move into other organs, including the spleen and the kidney, or even the brain (7). In this regard, Wang et al. confirmed that mitochondrial toxicity could play an important role in apoptosis created by CNTs (8). Mitochondria also have a significant role in oxidation, apoptosis, cell cycle regulation, cell growth, and metabolism (9). Changing the structure of mitochondria can thus induce a significant decrease in cellular metabolism and lead to cell death (10). It is definitely accepted that one of the main causes of damage to the kidney cells is elevated oxidative stress.

Moreover, studies in the related literature have shown that CNTs can result in inflammation, apoptosis, and cytotoxicity by inducing oxidative stress, thereby lowering cell viability (11). Nevertheless, no study was found on MWCNT-induced toxicity by oxidative stress in renal

mitochondria. Accordingly, this study was to investigate the effects of MWCNTs on oxidative stress as well as survival in mitotic mitochondria in rats.

CA is a natural phenolic compound found in coffee, some fruits, and traditional Chinese medicine (12). This substance and its analogues have a variety of pharmaceutical activities, i.e., anti-oxidant, anti-viral, anti-inflammatory, and anti-cancer properties (13). Therefore, CA was selected as an antioxidant to reduce oxidative stress produced by MWCNTs.

Materials and Methods

Materials

MWCNTs (>%95, OD: 5-15 nm), CA, and other chemicals, solvents, and reagents (Sigma Chemical Co.) were purchased from Pars Biochemistry Store (Ahvaz, Iran).

Animals

For the given tests, Wistar male rats (about 200-250 g) were received from Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, and all experimental methods were fulfilled in accordance with ethical protocols introduced by the Animal Ethics Committee at Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1396.45).

Preparation of Nanoparticles

Using distilled water, stoke suspension (600 mg/ ml) of MWCNTs was made. In order to provide a better dispersion of the particles, three drops of Triton X-100 were added to the volume and then it was shaken for 20 seconds using a vortex and subsequently dispersed using a Sonicator Probe (Ultra Sonic VCX-750 W model) at 4 degrees and 60 cycles for 30 minutes. The suspension was diluted with distilled water to make the desired concentration and then dispersed once again by a Sonicator Probe for 10 minutes (2).

Size and Morphology of Nanoparticles

Isolation of Mitochondria

The mitochondria of the tissues were isolated by differential centrifugation. First, the kidneys were cleaved into small pieces and then homogenized in mitochondria separation buffer (cold mannitol solution, 225 mM(and subsequently followed for separation of nuclei, unopened cells, and other noncellular tissues by centrifugation at 1500 xg and a temperature of 4°C for 10 minutes. The upper layers were centrifuged at 4°C at 12000 xg, for 10 minutes. After that, the upper layer was cast off, and the mitochondria were re-suspended in a separation medium (75 mM sucrose, pH 7.4, 0.2 mM

EDTA, and 0.225 M D-mannitol) and once again centrifuged for 10 minutes at 12000 xg. Ultimately, the mitochondria were suspended at 4°C in a Tris buffer with 0.05 M Tris-HCl, 0.25 M sucrose, 20 M KCl, 2.0 MM MgCl2, pH 4, and 1.0 mM Na2HPO4. In this study, mitochondria were newly provided for each test and were then used after 5 hours with the exception of mitochondria employed in ROS tests and MMP suspended in respiration buffer (10 mM Tris, 0.5 mM MgCl2, 5 mM sodium succinate, 0.32 mM sucrose, 50 µM EGTA, 0.1 mM KH2PO420, mM Mops), and buffer (68 mM D-mannitol, 220 mM sucrose, 10 mM KCl, 2 µM rotenone, 50 µM EGTA, 5 mM KH2PO4, 10 mM HEPES, 5 mM sodium succinate, and 2 mM MgCl2) (14, 15). To ensure high-quality mitochondrial separation, all steps were performed on ice.

Protein Measurements

Binding protein molecules to Coomassie brilliant blue and using a BSA as a standard, the mitochondrial protein concentration was established (16). To meet normalization in all the mitochondrial tests, mitochondrial samples were also utilized.

Dose Assessment of MWCNTs

In order to select the best dose, the given doses were exposed to mitochondria for one hour in an incubator based on previous studies (2, 19). To this end, the mitochondria were divided into 6 groups: (1) control (negative control); (2, 3, 4, 5 and 6) MWCNTs (20, 40, 80, 160, and 320µg/ ml). Then, the mitochondrial viability rate was measured using the MTT assay according to the reduction of yellow tetrazole to purple formazone due to mitochondrial respiration in live cells. Moreover, viability rate was shown as a percentage control, which is 100% assumed. After selecting the effective dose of nanoparticles, the mitochondria were divided into 6 groups: control group; mitochondria group exposed initially in the environment for 1 hour and then exposed to the CA (80 µM) and subsequently exposed to the environment for 1 hour; groups 3, 4, and 5, exposed to CA (160, 80, 40 and 20 µM) for 1 hour and then exposed to MWCNTs (320 µg / ml) for 1 hour; and group 6, exposed to MWCNTs for 1 hour (320 µg / ml) (17).

Determination of Complex II Activity using MTT Assay

The MTT assay is known as a sensitive and reliable indicator of the activity of mitochondrial complex II. In brief; for 1 minute, 1 ml of mitochondrial suspensions of the kidney was centrifuged in 10000 xg. Mitochondria were then suspended in 970 µl of isolation buffer and 500 µl of 0.5 µM MTT solution and incubated at 37°C for 45 minutes. Then; to dissolve the created purple formazan,

they were dissolved in 800 µl DMSO. The absorbance was also determined at 570 nm by means of a spectrophotometer (UV-1650 PC, Shimadzu, Japan) **(3, 18, 19)**.

Mitochondrial ROS Level Assay

Employing a fluorescent DCF-DA probe, the ROS rate in mitochondria was also measured. In sum, isolated mitochondria (protein 0.5 mg/ml) were washed with buffer phosphate (PBS) and incubated for 10 minutes with 1.6 mM DCM-DA at 37°C. Fluorescence was then measured via a fluorescence spectrometry of Perkin Elmer LS-50B (California, USA) at 500 and 520 nm emission and excitation wavelength, respectively (20, 21).

Measurement of Malondialdehyde Levels

Determining the thiobarbituric acid reactive substances (TBARS), MDA levels of renal mitochondria were assessed. To this end, 500 µl of mitochondrial suspension was added to 1.5 ml of TBARS (10%). Then, 2 ml of TBARS (0.67%) was mixed with the supernatant (1.5 ml) and placed for 15 minutes in a boiling water bath. Next, butanol (2 ml) was added, and then centrifuged (4000 xg in 15 minutes) after cooling the samples, and the absorbance was assessed at 535 nm. Using various dilutions of 1, 1, 3, 3-tetramethoxpropane, the standard MDA curve was prepared (22, 23).

Measurement of GSH Level

To determine the amount of GSH mitochondria; firstly, 5-sulfosalicylic acid (5%) was added to the samples. To remove the precipitated protein, the samples were centrifuged for 15 minutes at 13000 xg and 4°C, and subsequently, the upper layer was collected. Afterward, 5-Dithiobis (2-nitrobenzoic acid; DTNB) was added to the upper layer, and the total TNB was measured using a spectrophotometer at a wavelength of 412 nm (24).

Determination of CAT Activity

The CAT activity was investigated using L.Goth method. For this purpose, 500 µl Tris-HCl 0.05 mM, 1 ml H_2O_2, and 50 µl of mitochondrial suppression were mixed and incubated for 10 minutes, and then the reaction was blocked by adding 500 ml of 4% ammonium molybdate solution. It should be noted that the absorbance was read at 410 nm, and the result was expressed as protein U/mg (21).

MMP Measurement

Rhodamine 123 (Rh123) as the mitochondrial absorption of cationic fluorescent dye was utilized to evaluate the MMP. Rh123 is recognized as a lipophilic cation that can bind to parts of the mitochondrial membrane selectively and with a negative charge. Following the membrane hyperpolarization, the Rh123 enters the mitochondria and reduces fluorescence (Bindokas et al., 2003). The mitochondrial suspension (0.5 mg protein/ml) containing 10 µM Rh123 was reacted in MMP buffer. Then; using a Shimadzu RF-5000U Florescence Spectrophotometer, fluorescence was also examined in λEx=490nm and λEm=535nm (25, 26).

Statistical Analyses

Descriptive statistics, i.e., mean ± SD, were used to report the results. The statistical analyses were performed through one-way analysis of variance (ANOVA) using Prism Software (version 8). Using Tukey's post-hoc test, group differences were also calculated.

Results

Dose Optimization of M**WCNTs**

To select the best dose of MWCNTs, mitochondria were exposed to various concentrations of MWCNTs (20, 40, 80, 160, and 320 µg / ml) for 1 hour; after that, viability rate was tested using the MTT assay. The viability of the groups compared with H2O2 50 µM as control was also assumed to be equal to 100%. When mitochondria were exposed to 160 µg/ml of MWCNTs, reduction in viability was approximately 50% in comparison with mitochondria treated with 50 µM H2O2 ($p<0.01$). Therefore, 160 µg/ml of MWCNTs were used in the next experiments (Fig 1).

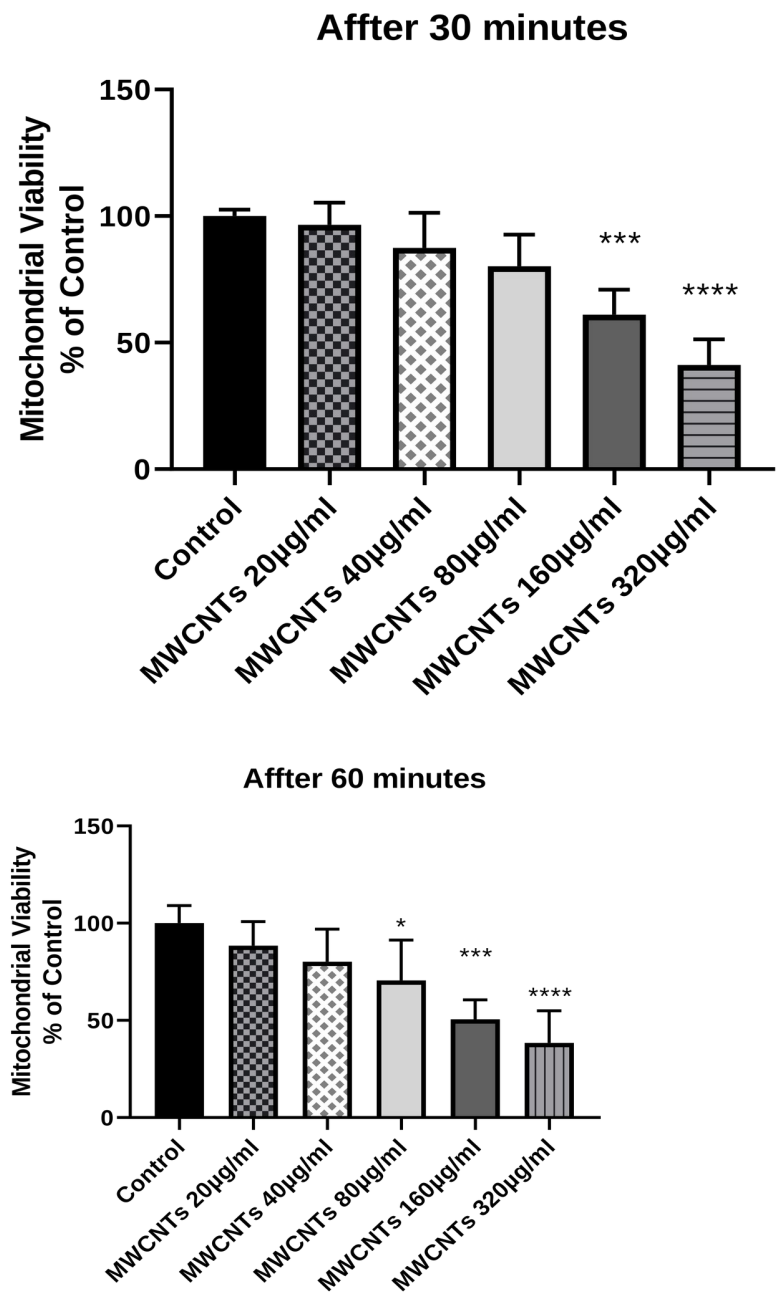


Figure 1; Effect of MWCNTs on the mitochondrial complex II after 30 and 60 min. Values are represented as mean ± SD and (n = 6). Difference between control and other groups is significant at $p < 0.01$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Effect of CA on Viability Rate of Mitochondria Exposed to MWCNTs

Cell viability was decreased to about 45% when the mitochondria were exposed to 160 µg/ml of MWCNTs for 1 hour. Then, the effect of different doses of CA on viability rate was investigated.

MTT assay showed that pre-treatment of mitochondria with 80 µM of CA could significantly increase the viability rate ($p<0.0001$) (See Fig 2). However, there was no significant change in cell viability in comparison with the control group following 1-hour exposure to 80 µM CA.

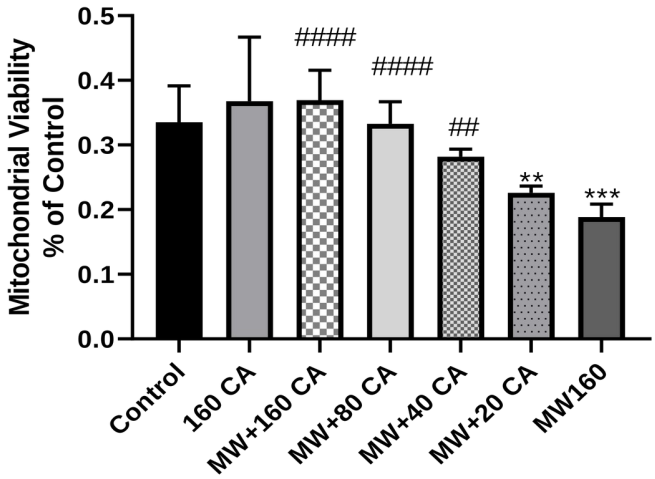


Figure 2: Effect of caffeic acid on cell viability in rat Kidney mitochondria exposed to MWCNTs. Results of treatment groups were compared with control group (** $P < 0.01$, *** $P < 0.001$) and MWCNTs 160 ($^{\#\#} P < 0.01$, $^{\#\#\#\#} P < 0.0001$). Values are expressed as mean ± SD.

Effect of CA on Oxidative Stress in MWCNT-Treated Mitochondria

The study findings showed significant ROS production as mitochondria were exposed to 160 mM of MWCNTs ($p<0.05$) (See Fig 3). However, pre-treatment with 80 µM of CA could significantly inhibit the production of ROS by MWCNTs ($p<0.0001$). Pre-treatment with 80 µM of CA did also show significant changes compared with that in the control group ($p<0.001$).

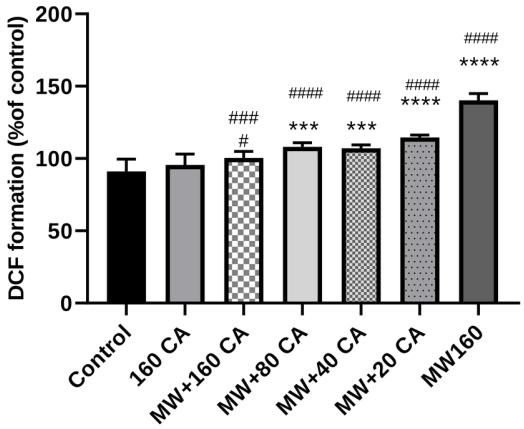


Figure 3: Effect of caffeic acid on ROS in rat Kidney mitochondria exposed to MWCNTs (dichlorodihydrofluorescein=DCF). Results of treatment groups were compared with control group (*** $P < 0.001$, **** $P < 0.0001$) and MWCNTs 160 ($^{\#\#\#\#}P < 0.0001$).

Values are expressed as mean ± SD.

Effect of CA on MDA Levels in Mitochondria Exposed to MWCNTs

After incubating mitochondria with 160 µg/ml MWCNTs, MDA level (nmol/mg protein) augmented significantly compared with that in the control group ($p<0.001$). Additionally, the 1-hour treatment of mitochondria with 40 µM of CA mitigated MDA level in comparison with that in the MWCNTs group (See Fig. 4).

Effect of CA on GSH in Mitochondria Exposed to MWCNTs

The results of GSH evaluation revealed a significant decline in MWCNTs group compared with that in the control group ($p<0.0001$). Additionally, 1-hour mitochondrial treatment with 80 µM of CA could prevent this reduction in GSH levels in mitochondria treated with MWCNTs ($p<0.001$) (See Fig 4).

Effect of CA on CAT Activity in Mitochondria Exposed to MWCNTs

According to the study findings, the activity of CAT in mitochondria (unit/mg protein) declined significantly after adding MWCNTs to the mitochondria compared with that in the control group ($p<0.0001$). Moreover, a significant increase was also reported in CAT activity after pre-treatment with 80 µM of CA ($p<0.01$) compared with the MWCNTs group (See Fig 4).

Effect of CA on MMP in Mitochondria Exposed to MWCNTs

The cationic fluorochrome R123 pigment was utilized to assess MMP. In this respect, MWCNTs could significantly lower MMP in mitochondria ($p<0.01$) (See Fig 4), but the 1-hour treatment of mitochondria with 80 µM of CA increased MMP compared with MWCNTs ($p<0.05$).

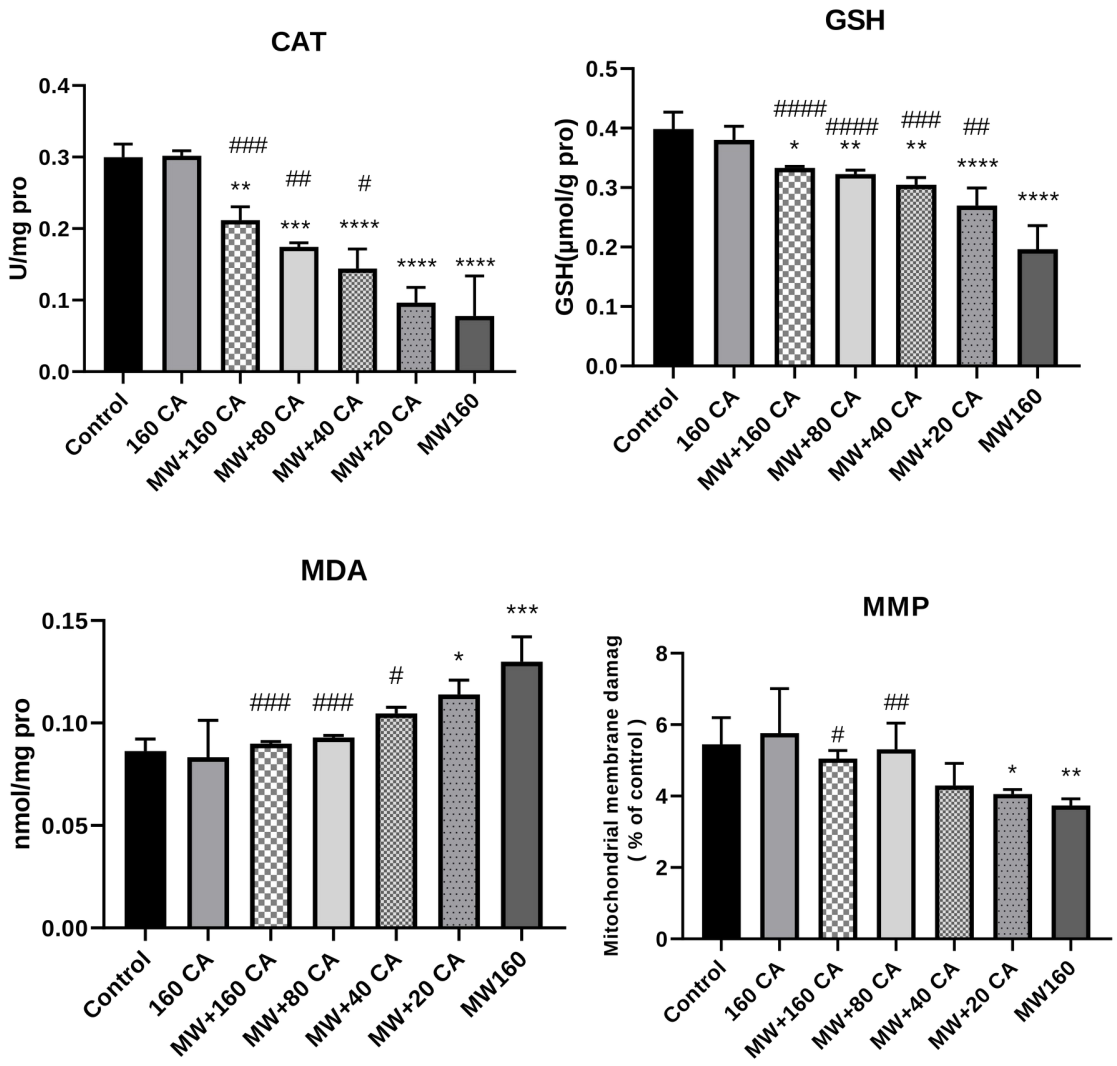


Figure 4: Effect of caffeic acid on the mitochondrial MDA, GSH and catalase activity in rat Kidney mitochondria exposed to MWCNTs. Results of treatment groups were compared with control group ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$) and MWCNTs 160 ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$). Values are expressed as mean ± SD.

Discussion

Mitochondria are dynamic organelles existing in numerous functions of metabolism and homeostasis of the living cells. Moreover, they play a central role in the generation of cellular energy by means of oxidative phosphorylation, calcium homeostasis, caspase-dependent apoptosis

initiation, as well as a cellular stress response (27). In this regard, it is required to find mitochondrial accuracy for special functioning of the oxidative phosphorylation system. Mitochondria have five complexes along with two electron carriers that are combined into the oxidative phosphorylation system to reduce ROS levels (28). To regulate the production of cellular energy, mitochondria are thus able to send signals (mitochondrial ROS (mtROS) and transmembrane potential) to other cell organelles. Accordingly, excessive mtROS production is responsible for cellular pathology that can directly affect bio-molecules (29). CNTs can also cause oxidative stress in cells. As reported in the related literature, CNTs can directly interfere with cell membranes, activate inflammasomes, and also lead to ROS production (30). Such research studies have further reported that MWCNTs cause tissue damage via ROS generation based on dose and time and consequently induce caspase 3 activity (29, 31). It has been even reported that MWCNTs have induced oxidative stress and reduced cell viability (32). In the present study, protective effects of CA in renal mitochondria in rats against MWCNTs-induced oxidative stress damage was investigated. The present study also reported that exposure of mitochondria to MWCNTs had diminished cell viability based on doses, and then cell viability had decreased up to 3 folds at the highest concentration (160 µg/ml). Correlated with ROS formation, there was also an increase in fluorescence in MWCNTs group. The contemporary treatment of CA, along with MWCNT similarly boosted viability in a dose-dependent manner and showed its protective effect above 80 µM of CA. In this study, the effects of MWCNTs on the MMP of samples were similarly examined. In this respect, MWCNTs decreased MMP, as cited in the study by Guo et al. demonstrating that MWCNT could lead to a drop in MMP of cells consistent with cellular apoptosis (33). In this study, MMP treated with MWCNTs was lower than that of mitochondria treated with CA.

Additionally; MDA, GSH, and CAT activity were investigated in all groups. The results of this study imply that MWCNTs can make significant changes in GSH activity and diminish GSH in renal mitochondria. Decreased CAT levels were also observed in the MWCNTs groups, likely because of the high concentration of MWCNTs (160 mg/kg) employed in this study.

Formed within oxidative stress conditions, MDA is known as a major peroxidation product that can be used as a lipid peroxidation indicator (34). It should be noted that the instability of plasma membrane originates from active oxygen atoms produced by peroxide (35). The ROS detoxification is also of importance for cell viability. If ROS are not detoxificated by antioxidant materials, peroxides are produced by lipid peroxidation, and toxic aldehyde such as MDA can

disrupt cell function (36). Nevertheless, significant rising trends were observed in mitochondrial MDA levels in the MWCNTs groups in this study, and MDA content in the MWCNTs was found to be higher than that in other groups. Together with the given changes in antioxidant enzyme activity, the MDA results indicated that the highest level of oxidative stress had occurred in renal mitochondria of MWCNTs-treated group.

It should be noted that CA is endowed with numerous biological activities, including ROS scavenging, immune stimulation, and lipid peroxidation quenching (37). The study results in this respect showed that antioxidant enzyme activities had significantly amplified in CA-treated groups compared with MWCNTs group, suggesting the anti- oxidative effect of CA on mitochondria. The major finding of this research is that CA showed a protective effect against MWCNTs-induced mitochondrial toxicity. Using reactive oxygen metabolites, CA can also protect biopolymers and reduce oxidative DNA damage (38). The probable mechanism of this effect is that CA can act by increasing both nuclear translocation of Nrf2 and expression of antioxidant enzymes, which in turn decreases oxidative stress (39). Previous studies in this domain revealed that CA administration to rats affected with breast cancer had improved macro-molecular structure including total protein content in the liver tissues, breast, as well as serums indicating maintained cell structure and integrity along with modulated nucleic acids. Furthermore, CA mitigates membrane-bound marker enzyme and peroxidation reactions and also increases the enzymic responses of the tricarboxylic acid cycle, adenosine triphosphatases, as well as antioxidants. These results indicate that mitochondria are a good candidate for the study of toxicity associated with high-dose MWCNTs.

Conclusion

To investigate toxicological effects induced by MWCNTs in rat mitochondria, a number of anti-oxidative defense system parameters were examined. According to the study results, antioxidant enzymes machinery could play an important role in coping with excessive ROS and protecting mitochondria against oxidative damage, even though they were inhibited following being exposed to the highest concentrations of MWCNTs. Besides, CA could reduce oxidative stress and improve antioxidant status in mitochondria. As a whole, there is a need to conduct further research studies to evaluate CA treatment efficiency against the effects of MWCNTs in animals.

Ethical approval: All experimental methods were fulfilled in accordance with ethical protocols introduced by the Animal Ethics Committee at Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1396.45).

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Authors' Contribution: M. S, L. Z & M. A.D designed the experiments. M. S, M.Sh & M.A. D carried out the analysis tests. S.A, L. Z & M. S prepared the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest.

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2 Revision 1

2 comments · 12 Feb 2023 – 15 Feb 2023

Referee 3 Reviewer

MINOR REVISION 12 Feb 2023

The work seems to have been competently carried out, and the results have been well interpreted. It is of sufficient quality to be published in the Jundishapur Journal of Natural Pharmaceutical Products with brief corrections. In its current state, the level of English throughout your manuscript does not meet the journal's desired standard. There are a number of grammatical errors and instances of badly worded/constructed sentences. Please check the manuscript and refine the language carefully. Probably the editorial office could help the authors in this respect.

Comment 1: There are numerous writing errors (most of the chemical formulas; nanomaterial's in the first line of the introduction; and so on).

Comment 2: Improve some sentences in the various parts of the manuscript. For example:
Introduction section: The sentence “Studies in the related literature have” is repeated two times in the text, while better sentences can be used.
Discussion section: Such research studies have further reported that MWCNTs cause tissue damage via ROS generation based on dose and time and consequently induce caspase 3 activity.

Comment 3: The word studies is not very appropriate and has been used too much in the manuscript. Related sentences should be improved.

Comment 4: Introduction section: It is better to use new references (last 3 years) in the literature review.

Comment 5: Preparation of Nanoparticles section; in line 4, do you mean 4 different temperatures or 4 degrees Celsius (4 °C)?

Comment 6: Preparation of Nanoparticles section: The phrase “were added to the volume” should be replaced with a better one.

Comment 7: There must be a space between the numbers and their units, and attention must be paid to subscripts and superscripts. For example: change 4°C to 4 °C.

Comment 8: Isolation of Mitochondria section: Please check the concentration of KCl and MgCl2. Millimolar is represented by mM, not MM.

Editor-in-Chief

MINOR REVISION 15 Feb 2023

Dear Authors,

Your revised manuscript needs minor revision. Please correct it according to the reviewer's comments and upload the corrected version with a point-by-point response to the reviewer. Remember to highlight the changes in the revised manuscript.

Kind regards,
JJNPP EIC

3

Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Comment 1: There are numerous writing errors (most of the chemical formulas; nanomaterial's in the first line of introduction; and so on).
The text was edited.
Comment 2: Improve some sentences in the various parts of manuscript, For example:
Introduction section: Sentence "Studies in the related literature have" is repeated two times in the text while better sentences can be used.
Discussion section: Such research studies have further reported that MWCNTs cause tissue damage via ROS generation based on dose and time and consequently induce caspase 3 activity.

The sentences were corrected.

Comment 3: The word studies is not very appropriate and has been used too much in the manuscript. Related sentences should be improved.

The sentences were corrected.

Comment 4: Introduction section: It is better to use new references (last 3 years) in literature review.
A new reference was added.

Comment 5: Preparation of Nanoparticles section; in line 4, do you mean 4 different temperatures or 4 degree of centigrade (4 ºC)?

It was corrected

Comment 6: Preparation of Nanoparticles section: The Phrase "were added to the volume" should be replaced with better one.

It was corrected.

Comment 7: There must be a space between the numbers and their units and attention to subscript and superscripts. For example: 4°C change to 4 °C.

It was corrected

Comment 8: Isolation of Mitochondria section: please check the concentration of KCl and MgCl2. Millimolar is represented by mM not MM

It was corrected

3 Revision 2

1 comment · 19 Mar 2023

Mohammad Panahimehr Reviewer

ACCEPT 19 Mar 2023

In its current state, your revised manuscript will be accepted for publication in the Jundishapur Journal of Natural Pharmaceutical Products.