International Journal of Pharmaceutical and Bio-Medical Science

ISSN(print): 2767-827X, ISSN(online): 2767-830X

Volume 04 Issue 10 October 2024

Page No: 816-825

DOI: https://doi.org/10.47191/ijpbms/v4-i10-06, Impact Factor: 7.792

Effects of Functionalized Carbon Nanotube on Cellular Homeostasis of Murine Hepatocytes

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ABSTRACT

Nanotechnology in medicine represents a revolution offering significant advancements in the diagnosis, treatment, and prevention of diseases. This technology enables the development of devices and materials at the nanometer scale, allowing precise interactions with biological systems at the molecular and cellular levels. Among various nanomaterials, carbon nanotubes (CNTs) stand out due to their unique physical, chemical and mechanical properties. Due to their high strength, electrical and thermal conductivity and large surface area, CNTs have a wide range of applications. In healthcare they are explored for drug delivery and imaging diagnostics. CNTs can be functionalized to target drugs directly to diseased cells, minimizing side effects and enhancing treatment efficacy, additionally can be used in biosensors for early disease detection and in tissue engineering to cellular regeneration. The present study evaluated the effects of OCNT-TEPA, a multi-walled carbon nanotube functionalized, in murine hepatocytes AML-12. Cells were exposed to different concentrations of the sample for 12, 24, 48, and 72 hours. Cellular metabolism tests, cellular morphology by optical microscopy, synthesis of reactive oxygen species, changes in membrane potential and IL-6 cytokine secretion were performed. The results show that OCNT-TEPA altered the homeostasis of hepatocytes, as cellular metabolism decreased, cellular morphology was altered, the membrane experienced changes in its electrical potential, oxidative stress increased and inflammatory signaling molecules were synthesized. This alteration is dose-dependent, which may be harmful to hepatocytes at high concentrations but could be applied to the body at lower concentrations without causing harm.

KEYWORDS: Carbon Nanotube; Nanoparticles; Cytotoxicity; Cellular viability; Oxidative stress; Murine hepatocytes.

ARTICLE DETAILS

Published On:
10 October 2024

Available on:
https://ijpbms.com/

1. INTRODUCTION

Nanotechnology is a major scientific field responsible for developing and manipulating devices, materials, and molecules at a scale ranging from 1 to 100 nanometers, the nanometric scale. Due to this small size, precise mediation at cellular and molecular levels is possible. This is important as medicine has been experiencing significant advancements due to nanotechnology, as it offers modern, simple, and innovative solutions for disease prevention, treatments, and diagnostics of [1].

A nanotechnological advancement that has propelled medicine is the specific and targeted delivery of molecules and drugs. An example is the use of carbon nanotubes (CNTs), which are employed for this purpose (REVATHI, 2015). CNTs have carbon atoms arranged in a hexagonal pattern and exhibit a cylindrical, nanometric shape. They are distinguishable into two types: single-walled, known as SWCNTs, and multi-walled, known as MWCNTs. This structure imparts unique and exclusive characteristics and properties [3].

CNTs are ideal and important in industry and healthcare due to their wide range of applications, as they have high electrical and thermal conductivity and mechanical strength [4,5]. In the industrial process, CNTs are used to optimize the durability and strength of materials, create more efficient and faster electronic components, and develop materials with improved electrical and mechanical properties [6,7].

In healthcare, CNTs have a significant impact in various areas. For drug delivery, CNTs can be functionalized to carry and release medications directly into specific cells, minimizing side effects and increasing the effectiveness of treatments [8]. This is particularly useful in cancer treatment, where targeted delivery can ensure that medications exclusively reach tumor cells while preserving healthy cells [9]. Additionally, CNTs can be used in photothermal therapy, where they are employed to heat and destroy cancer cells upon exposure to light, offering a less invasive approach to treatment [10].

This study explores the use of a carbon nanoparticle called OCNT-TEPA. The production of this nanoparticle involved adding a tetraethylenepentamine (TEPA) ligand to its surface, after synthesizing the nanoparticle from an oxidized MWCNT. The inclusion of this ligand enables the creation of a nanofluid with greater thermal stability, improved viscosity, and resistance to both high temperatures and salinity [11]. This modification gives the nanoparticle more advantageous characteristics for applications, as nanofluids are widely used in the oil and gas industry [12] and the exposure of workers to this material is still not elucidated by science.

In summary, nanotechnology and carbon nanotubes represent an advanced frontier in science that is transforming medicine and industry. The unique properties of CNTs offer new opportunities to enhance the efficiency and effectiveness of medical treatments, while also driving innovations in materials and industrial technologies. Nanotechnology holds the promise of enriching and advancing human health by improving quality of life, as its applications are continuously expanding [13,14].

In this context and considering the scarcity of research on the OCNT-TEPA nanoparticle, concerns arise about the potential toxic effects resulting from the modification of the carbon nanotube with the TEPA polymer. Functionalization can influence the surface area, conformation, and physicochemical properties of the nanotube, making it essential to evaluate potential adverse effects [11]. Therefore, it is crucial to investigate the potential cytotoxic effects of OCNT-TEPA in biological models. This study specifically examines the cytotoxic profile of OCNT-TEPA when exposed to murine hepatocytes AML-12. Changes in cellular metabolism, activation of oxidative, inflammatory, and morphological processes were analyzed. The data from these analyses may help understand the effects of ONCT-TEPA on cells and infer its safety and efficacy.

2. MATERIAL AND METHODS

2.1 OCNT-TEPA nanoparticle

Petrobras (Brazil) supplied the OCNT-TEPA sample. The corporation indicates that the particles have lengths that range from 2 to 10 μm and diameters between 7 and 20 nm.

2.2 Characterization

The materials were examined using transmission electron microscopy (TEM) and high-resolution TEM (HR-TEM) at 200 kV using a Jeol 2100F microscope. The TEM sample was prepared by dripping an aqueous solution over a copper grid that had been coated with carbon. The grid was then let to dry at room temperature. The Inspect F50 microscope was used to perform scanning electron microscopy (SEM) at 5 kV. For SEM analysis, an aqueous solution containing the particles was dropped onto a silicon substrate.

2.3 In vitro assays

The AML-12 cell line (ATCC - AMERICAN TYPE CULTURE COLLECTION, [s.d.]) of murine hepatocytes was employed. Dulbecco's Modified Eagle's Medium, Sigma-ALDRICH, USA) was used to cultivate the cells. It was supplemented with 1% and 10% of the antibiotics streptomycin/penicillin and serum fetal. 90% confluency was obtained by the cells under subculture conditions, which involved keeping them in a humidified chamber at 37 °C and 5% CO2. Initially, 1000, 750, 500, 250, 100, 50, 10, and 1 μg/mL were the investigated sample concentrations, determined by an EC50 study. These concentrations were then dissolved in the whole DMEM medium. There was also an untreated cell group included in the control group (Control). The experiments were performed in octuplicate

(N=8) as part of three independent experiments, involving exposure to different material concentrations for 12, 24, 48 and 72 hours. The next assays were performed only in 24 hours and with de concentrations of 1, 50, 250, 500 and 1000 µg/mL. The assessments conducted included cell viability through analysis of mitochondrial function [15] and membrane integrity [16], detection of reactive oxygen species (ROS) [17], mitochondrial membrane potential by flow cytometry with rhodamine 123 [18], quantification of IL-6 in the cell culture supernatant by ELISA (BD OPTEIA TM MATERIALS PROVIDED) and examination of cell morphology using optical microscopy. Detailed experimental procedures for these techniques are provided in the Supplementary Information.

3. RESULTS AND DISCUSSION

3.1 Characterization

The samples' structural and morphological properties were examined. **Figure 1A** illustrates SEM images wich shows the morphological uniformity of the OCNT-TEPA. **Figure 1B** illustrates TEM images. Detailed observations of OCNT-TEPA were made using HR-TEM images (**Figure 1C-D** and **Figure 2A-B**).

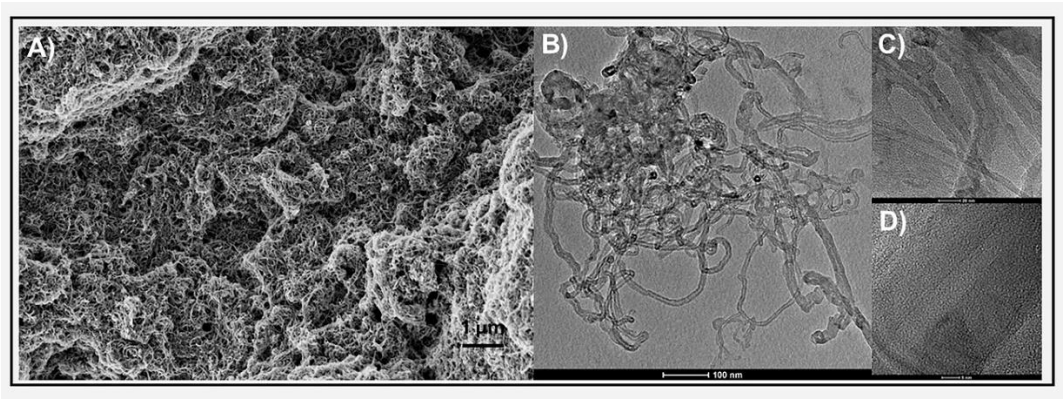

Figure 1. Images of the OCNT-TEPA sample. (A) SEM images (B) TEM images (C) HR-TEM images.

The OCNT-TEPA carbon nanotube was also previously characterized through scanning and transmission electron microscopy analyses to observe its size and performance in the presence of water and culture medium. FE-SEM analyses show the nanotube in water with uniform morphologies, a low degree of agglomeration, and an average diameter of 12.7 ± 3.0 nm, which is consistent with the manufacturer's information. However, in contact with the culture medium, agglomerations occur at all concentrations. TEM images reveal an internal diameter of 6 ± 1.8 nm, which increases when in contact with the culture medium, confirming the aggregation. Additionally, more than one layer is observed in its walls, characterizing them as multi-walled (MWCNTs) (DE GODOY et al., 2021, 2022).

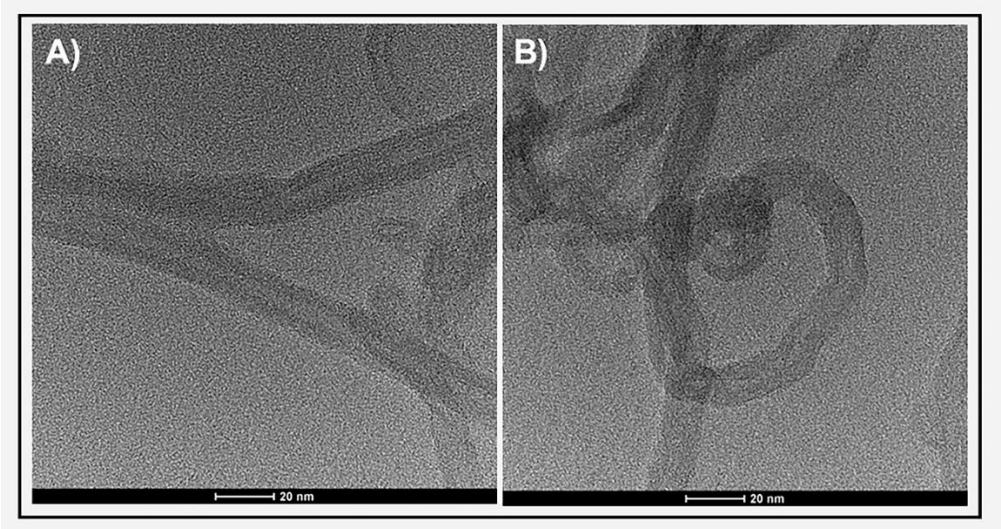

Figure 2. Images of the OCNT-TEPA sample. (A) HR-TEM images. (B) HR-TEM images.

The surface charge of OCNT-TEPA was measured by zeta potential in previous studies. In water, the zeta potential was approximately -13.3 ± 1.59 mV, indicating a negative charge due to carboxyl groups on the surface of the MWCNT, even after modification with TEPA. In the culture medium, the zeta potential showed an additional decrease in negative charge due to the formation of a protein corona, changing from -12.5 ± 1.05 mV to -9.21 ± 1.24 mV in the medium with 10% [20].

Previous studies examined the formation of a corona on the particle surfaces. The sample was incubated in the culture medium for 24 and 48 hours, and the size was

measured using DLS technique. For the sample in water, the value was 128 ± 11 nm, indicating some aggregation. After 24 and 48 hours of incubation, the DLS values increased significantly to 694 ± 48 and 729 ± 88 nm, respectively. After washing with water, the sizes remained larger, at 565 ± 38 and 624 ± 22 nm[11]. This reinforces the evidence of aggregation due to the corona, as observed in previous studies, altering the physicochemical properties and biological behavior of the particles [21].

3.2 In vitro assays

After characterizing OCNT-TEPA through imaging, the behavior of the nanotube was analyzed in murine hepatocytes from the AML-12 cell line to understand its impact on dynamic living systems. Initially, cellular metabolism was quantified as a percentage using the MTT salt, which is converted into formazan crystals by mitochondrial enzymes, producing a colorimetric reaction (MOSMANN, 1983). Higher absorbance indicates greater crystal formation and, consequently, balanced cellular metabolism. Additionally, neutral red (NR) dye was used, which penetrates the plasma membrane of healthy cells and accumulates in lysosomes, with higher absorbance indicating more efficient cellular metabolism [16].

The cellular metabolism kinetics were assessed (analysis at 12, 24, 48, and 72 hours) using the MTT salt assay (**Figure 3**). It is observed that there is a decrease in cellular metabolism with a significant difference starting from a concentration of 50 µg/mL at all four time intervals. After 12 hours of exposure to OCNT-TEPA, cellular metabolism shows higher % values (**Figure 3A**) compared to the other time intervals.

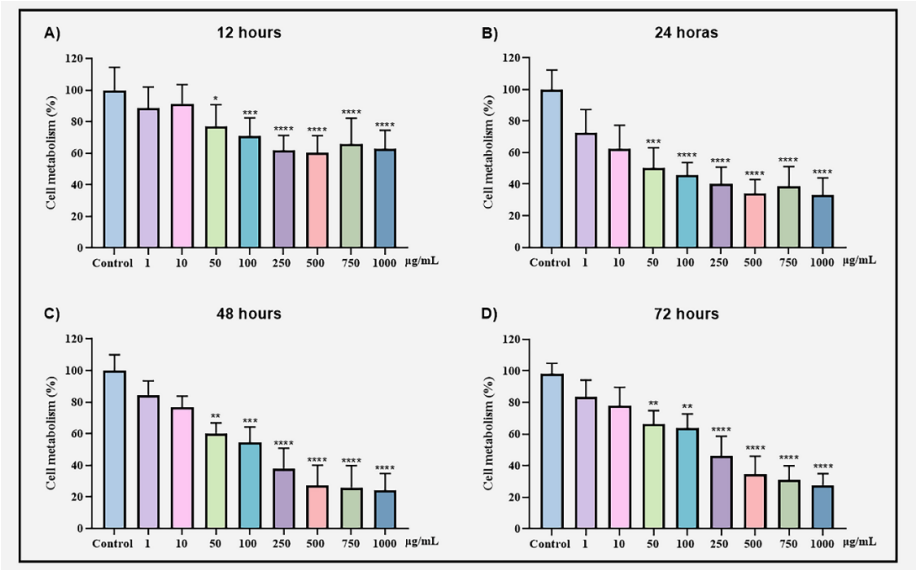

Figure 3. Results of MTT assay for the sample A) 12 hours. B) 24 hours. C) 48 hours. D) 72 hours. (*) *vs* Control: * p ≤0.05; ** p ≤0.01; *** p ≤0.001; ****p ≤0.0001. The results were presented as the median with the upper and lower quartiles: Me [Q1; Q3].

This percentage changes as the exposure time increases, as shown in **Figure 4A**. The 24 and 48-hour exposure intervals exhibit the lowest % values of cellular metabolism. **Figure 4B** displays the EC_{50} value of 24.58, at which concentration there is already a decrease in cellular metabolism.

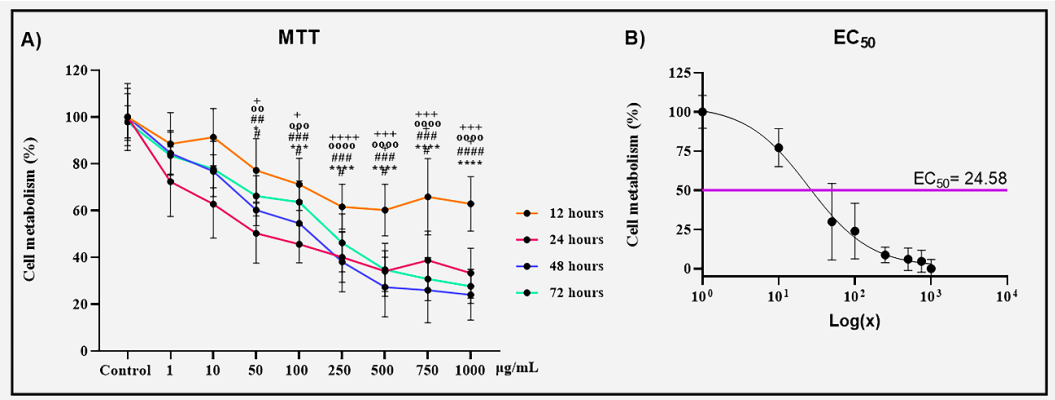

Figure 4. Results of MTT assay and EC_{50} for the sample A) MTT for 12, 24, 48 and 72 hours. B) EC_{50} for the sample: 24.58. (*) *vs* Control: * p ≤0.05; ** p ≤0.01; *** p ≤0.001; ****p ≤0.0001. The results were presented as the median with the upper and lower quartiles: Me [Q1; Q3].

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DOI: https://doi.org/10.47191/ijpbms/v4-i10-06, Impact Factor: 7.792

To better analyze the behavior of OCNT-TEPA, we observed the cellular metabolism of hepatocytes after 24 hours of exposure using the MTT salt and neutral red dye (**Figure 5**). The percentage of cellular metabolism with the MTT salt significantly decreases starting at a concentration of 50 µg/mL (**Figure 5A**), while with the neutral red dye, the decrease is significant starting at 250 µg/mL (**Figure 5B**).

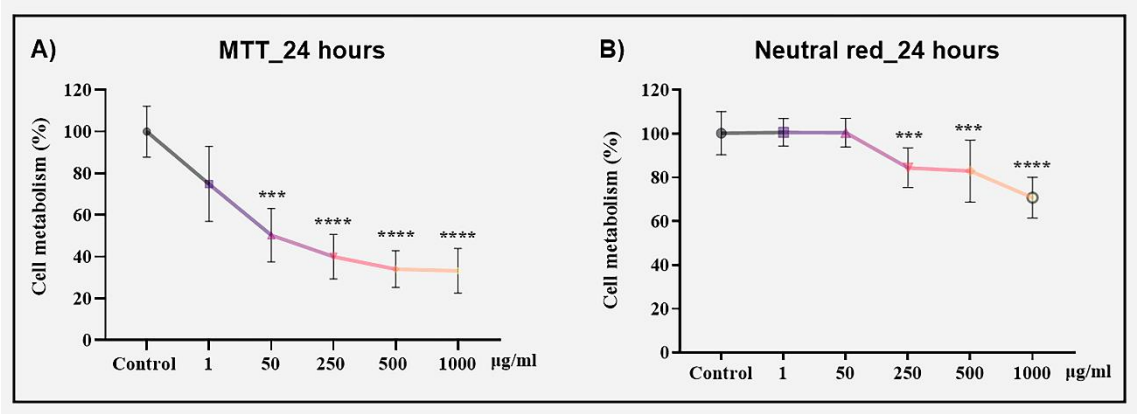

Figure 5. Results of MTT and neutral red. A) MTT_24 hours. B) Neutral red_24 hours. (*) *vs* Control: * p ≤0.05; ** p ≤0.01; *** p ≤0.001; ****p ≤0.0001. The results were presented as the median with the upper and lower quartiles: Me [Q1; Q3].

These findings are corroborated by optical microscopy images (**Figure 6**). In the Control group, the cell morphology is preserved, with intact plasma membranes. At a concentration of 1 µg/mL, smaller cells begin to appear with undefined membranes. At 50 µg/mL, the cells show alterations, and these changes become more pronounced with increasing concentrations of OCNT-TEPA.

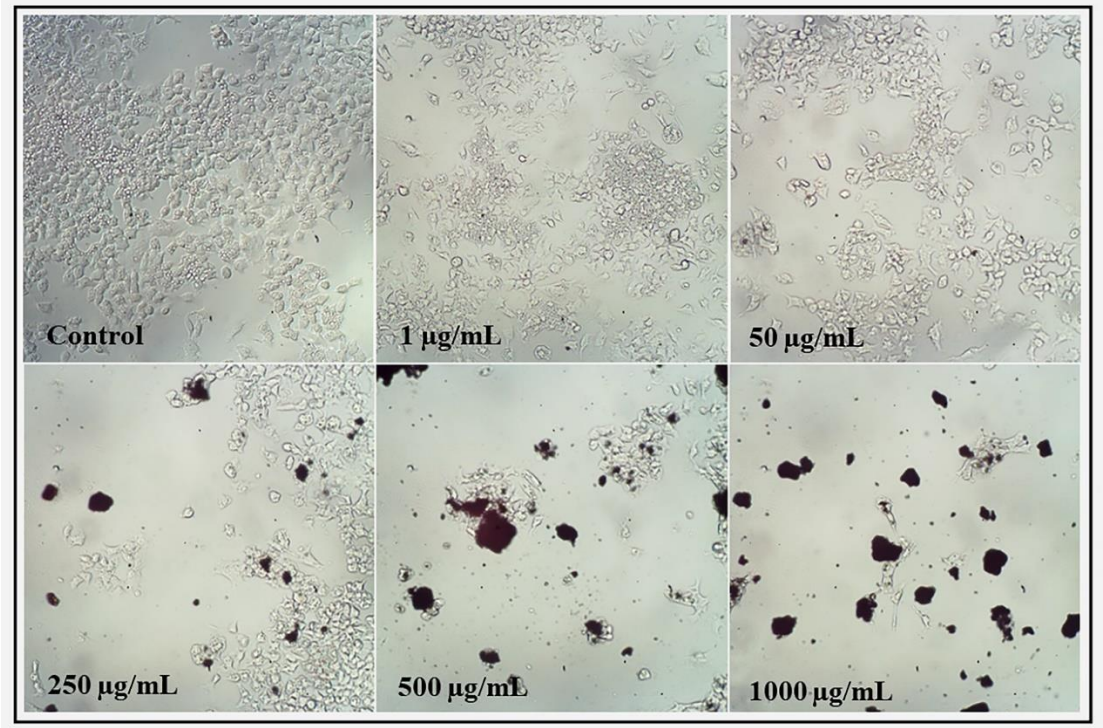

Figure 6. Cellular morphology of AML-12 hepatocyts after exposure to the sample final resolution 100X.

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AML-12 is a highly recommended liver cell for medium, biological, and pharmacological research, as it provides insights into liver function and the toxicity of any substance, and consequently, the progression of disease. This is essential for understanding how drugs are metabolized and whether they can cause liver damage[22] , especially since the effects of exposure to OCNT-TEPA are not yet known in medicine.

OCNT-TEPA has previously been exposed to murine fibroblasts (3T3 cell line)[20] and murine macrophages (J774 A1 cell line)[11]. Both studies, using the MTT salt and neutral red dye assays, demonstrated that the sample significantly decreases cellular metabolism and alters cellular morphology.

Such findings are similar to the effect of high concentrations of MWCNTs on macrophages (MH-S and raw264.7 cell lines), where cellular viability was compromised [23]. Nahle et al. also observed cytotoxicity of CNTs using rat alveolar macrophages (NR8383) in cell viability experiments [24]. Several authors recommend these methodologies within nanotoxicology, provided that an appropriate cell line, such as the one used in this study, is employed [25].

Cytotoxicity (reduced cellular metabolism and morphological changes) is related to the interaction of CNTs within the cellular environment, which can occur in various ways (such as endocytosis, phagocytosis, or needle-like penetration), potentially triggering alterations in signaling and cell cycle regulation[26,27] , thereby disrupting homeostasis. OCNT-TEPA has shown to interact with hepatocytes, causing damage to these cells.

Vuković et al. (2009) emphasize the connection between cytotoxicity and reduced proliferative activity of L929 fibroblasts when exposed to two different types of MWCNTs, one unmodified and one functionalized with amino groups (DETA, TETA, HAD, and PDA) [28]. Additionally, high concentrations of carbon nanotubes lead to nanoparticle accumulation forming clusters, which vary in size and make these concentrations more toxic, as observed in the OCNT-TEPA characterization analyses.

Moreover, from a physicochemical perspective, this cytotoxicity is directly influenced by the type of nanoparticle, size, composition, surface charge, morphology, porosity, aggregation and solubility. (HOLSAPPLE et al., 2005; KHAN; SAEED; KHAN, 2019).

OCNT-TEPA increased in size due to the corona effect, as shown by the results obtained using the DLS technique [11]. Larger nanoparticles tend to be more rapidly captured by the liver's reticuloendothelial system (RES), which can reduce the efficacy of drug delivery and distribution. (M. D. D'ISCHIA et al, 2020). Larger nanoparticles may also accumulate on the cell surface or in specific intracellular compartments. [32]. The size of OCNT-TEPA in the medium is 565 ± 38 and 624 ± 22 nm. Therefore, we believe that the complete penetration of the aggregated nanotube into the hepatocyte is hindered, as also observed in the optical microscopy images.

The penetration of CNTs through the cellular lipid membrane induces oxidative stress, production of free radicals, protein damage, genetic material compromise, and inflammation [33–35]. **Figure 7** illustrates how OCNT-TEPA was able to alter the mitochondrial membrane potential, increase ROS synthesis in hepatocytes, and enhance the secretion of the inflammatory cytokine IL-6.

Rhodamine 123 is a cationic fluorochrome (positive charge) that is highly attracted to the negative charge of the mitochondrial membrane, accumulating inside this organelle. OCNT-TEPA has a negative surface charge of -12.5 ± 1.05 mV in water, but in the medium, this negative charge decreases to -9.21 ± 1.24 mV. This reduction may explain the interaction of the nanoparticle with the membrane, altering its electric potential, as shown in **Figure 7A**. Additionally, higher concentrations of the sample result in greater interaction with the membrane.

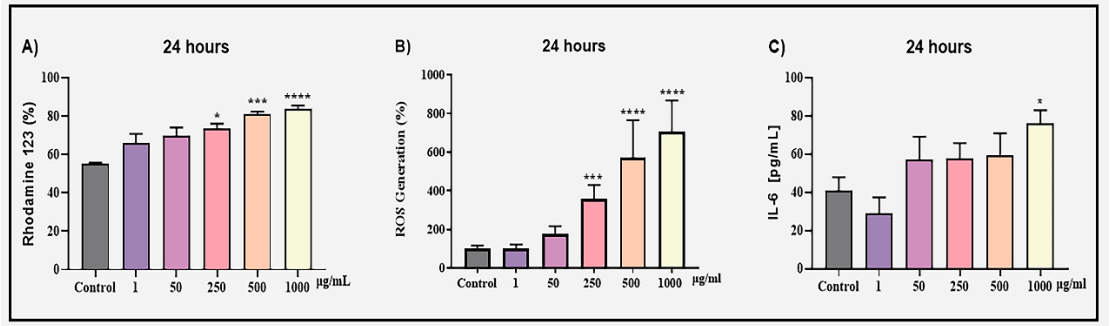

Figure 7. Results of Rhodamina 123, ROS and IL-6. A) RhB 123 fluorescence percentage, (B) ROS percentage, (C) IL-6 levels (*) *vs* Control: * p ≤0.05; ** p ≤0.01; *** p ≤0.001; ****p ≤0.0001. The results were presented as the median with the upper and lower quartiles: Me [Q1; Q3].

OCNT-TEPA altered the electrical potential of the mitochondrial membrane in J774 A1 macrophages [11]Possible changes in the mitochondrial membrane potential (MMP) of lung cell lines exposed to CNTs were evaluated and there was a change in the potential over a period of 1 to 7 days [36]. It is widely known that CNTs have the ability to alter the mitochondrial membrane potential because there is synthesis and release of excess ROS, changes in the release of cytochrome C, what triggers the cellular apoptosis activation pathway [36,37], and in the complexes of the mitochondrial electron transport chain [38].

Cellular components such as DNA, proteins, and lipids are damaged when oxidative stress occurs via excess ROS, such as free radicals (e.g., hydroxyl radicals, superoxide) and peroxides (e.g., hydrogen peroxide) [39]. OCNT-TEPA significantly increased ROS synthesis in hepatocytes **(Figure 7B)** starting at a concentration of 250 µg/mL, with this synthesis rising as the concentration of the nanotube increased. Godoy et al. demonstrated that both fibroblasts and macrophages experienced oxidative stress, as the cells produced very high levels of ROS following exposure to the nanotubes OCNT-TEPA (DE GODOY et al., 2021, 2022). Studies tested CNTs in RAW murine macrophages and observed an initial increase in ROS production, followed by a decrease after prolonged exposure to the CNTs, characterizing oxidative stress as a rapid and transient process [40]. Zhou *et al*. (2017) It also demonstrated the presence of ROS by using human lung A549 cells exposed to three different carbon nanotubes, which produced ROS at a concentration of 20 mg/mL in all three MWCNTs [41].

CNTs can increase the synthesis of reactive oxygen species (ROS) by cells through several mechanisms [42]. The formation of ROS on the surface of CNTs occurs through direct interaction with oxygen molecules [43]. Nanotubes can catalyze reactions that generate free radicals and other reactive species. CNTs may produce free radicals through interactions with molecular oxygen or due to chemical reactions on their surface. Superoxides and peroxides can be formed via these reactions [44]. By suppressing the activity of antioxidant enzymes like catalase and superoxide dismutase (SOD), CNTs can disrupt the cellular antioxidant system and increase the formation of reactive oxygen species (ROS) [43]. As part of the immunological response, exposure to CNTs can activate macrophages and cause an inflammatory response, which in turn produces ROS [45] Lastly, because of malfunctions in the electron transport chain, CNTs might stress out mitochondria and enhance the generation of ROS [46].

The OCNT-TEPA increased IL-6 levels in hepatocytes (Figure 7C). The sample also elevated the levels of this cytokine, as well as TNF, in J774 A1 macrophages [11,20]. Elevated levels of IL-6 are further indicative of oxidative stress, which can lead to cellular death [47,48].

As was previously indicated, the presence of CNTs can cause inflammatory reactions in cells, which in turn causes activated immune cells to produce ROS [45].

ROS generation may rise when inflammatory pathways like IL-6 are activated. Physical, chemical, and biological interactions with cellular components result in higher generation of ROS by CNTs, causing oxidative stress that can harm cells and contribute to a variety of diseases [47–49].

Additionally, CNTs are important for cellular regeneration and tissue engineering. By offering structural support and encouraging the regeneration of injured tissues, they can be employed as scaffolds for the development of new cells and tissues. This could completely change how degenerative illnesses and injuries are treated by providing new avenues for tissue replacement and repair [50,51]. Osteoblasts and fibroblasts were exposed to CNTs, resulting in a decrease in IL-6, which indicates biocompatibility [52] since this cytokine is one of the most prominent biomarkers for inflammation [53].

4. CONCLUSIONS

Carbon nanotubes have a wide range of applications due to their unique properties, such as high strength, electrical conductivity, and functionalization capabilities. They are used in various fields including electronics, computing, engineering, industry, energy, biotechnology, and medicine. Applications include biosensors, drug delivery carriers, and imaging techniques. The OCNT-TEPA investigated in this study is a recently synthesized functionalized nanotube that has proven to be highly cytotoxic at concentrations above 50 µg/mL, causing oxidative stress, decreased cellular metabolism, and increased synthesis of inflammatory cytokines, leading to cellular homeostasis imbalance. However, at lower concentrations, no cell damage was observed. Given this information, it is undeniable that conducting research on nanoparticle toxicity before their widespread use in any application is crucial.

ACKNOWLEDGMENTS

This study was supported by Petrobras (Process No 2017/00010-7). M.A. was supported by the São Paulo Research Foundation (FAPESP) 2023/08525-7. B. D. L. F. was supported by the São Paulo Research Foundation (FAPESP) (2013/07296-2 and 2022/06210-6). The authors would like to acknowledge to Dr. Márcia Regina Cominetti (Department of Gerontology, Universidade Federal de São Carlos, São Carlos, SP, Brazil) and Dr. Heloisa Sobreiro Selistre de Araujo (Department of Physiological Sciences,

Federal University of São Carlos, São Carlos, SP, Brazil) for the availability of the cytometer and fluorescence reader, respectively.

CREDIT AUTHORSHIP

K.F.G.: Conceptualization, Methodology, Investigation, Data curation, Analysis, Validation, Resources, Funding, Writing - original draft, Writing - review & editing. **K.F.G., J.M.A.R., B.L.D.F., M.A. and E.L.:** Methodology, Investigation, Data curation, Analysis, Validation. **K.F.G. and B.D.L.F.:** Writing - original draft, Writing - review & editing. **F.F.A.:** Supervision, Funding acquisition, Writing - review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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