International Journal of Pharmaceutical and Bio-Medical Science

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

Volume 02 Issue 12 December 2022

Page No: 679-686

DOI: https://doi.org/10.47191/ijpbms/v2-i12-16, Impact Factor: 5.542

Carbon Black CB-EDA Nanoparticles in Hepatocytes: Changes in the Oxidative Stress Pathway

Joice Margareth de Almeida Rodolpho[1], Krissia Franco de Godoy[2], Patricia Brassolatti[3], Bruna Dias de Lima Fragelli[4], Luciana Camillo[5], Cynthia Aparecida de Castro[6] , Camila Tita Nogueira[7], Marcelo Assis[8], Carlos Speglich[9], Elson Longo[10], Fernanda de Freitas Anibal[11]

[1,2,3,4,5,6,11]Laboratório de Inflamação e Doenças Infecciosas, Departamento de Morfologia e Patologia, Universidade Federal de São Carlos, São Carlos, Brazil,

[8]Department of Analytical and Physical Chemistry, University Jaume I (UJI), 12071, Castelló, Spain,

[8,10]Centro de Pesquisa Leopoldo Américo Miguez de Mello CENPES/Petrobras, Rio de Janeiro, Brazil,

[9]Centro de Desenvolvimento de Materiais Funcionais, Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil.

[7]PhD in Biotechnology

ABSTRACT

Background/Aims: Carbon Black (CB) is the most widely produced and commercially used nanocarbon. Growing evidence links nanomaterials to adverse effects, arising from their large surface area capable of interacting with biological systems. Due to the variety of applications and human exposures to nanoparticles, it is important to assess the potential health risk and understand the underlying mechanisms of their toxicity. The present study aims to investigate the cytotoxic effect of the Carbon black nanoparticle covalently linked to ethylenediamine (CB-EDA) on AML-12 cells, a lineage of murine hepatocytes.

Methods: For this, the cells were exposed to CB-EDA for 24h, at different concentrations of the nanoparticle (1, 50, 250, 500 and 1000 μg/mL). Effects on cell viability were evaluated using MTT and neutral red dye tests and analysis of changes in cell morphology. Furthermore, the oxidative profile (levels of reactive oxygen species - ROS and nitrogen - NOS) and inflammatory profile (production of IL-6 and TNF-α) were investigated.

Results: The results show that CB-EDA nanoparticle causes a reduction in cell viability at concentrations of 250, 500 and 1000 μg/mL after the exposure period. There was a significant increase in the production of ROS, NOS and pro-inflammatory interleukin TNF-α.

Conclusion: The data suggest that the CB-EDA nanoparticle has a cytotoxic potential in AML-12 hepatocytes, evidenced by the induction of oxidative stress and secretion of inflammatory cytokines, with a consequent decrease in cell viability.

KEYWORDS: Carbon black (CB) nanoparticles, AML-12 cells, reactive oxygen species, cell viability, oxidative stress.

ARTICLE DETAILS

Published On:
23 December 2022

Available on:
https://ijpbms.com/

INTRODUCTION

Nanotechnology is a branch of science that encompasses any technology operating on a nanoscale that has practical applications, employing individual atoms and molecules to form functional structures. The field of nanotechnology involves the creation and use of chemical, physical and biological systems with structural characteristics between individual atoms or molecules in submicromic dimensions, and the assimilation of resulting nanostructures into larger systems. The simplest definition of nanotechnology is "nanoscale technology", whose components and structures

represent significantly improved properties, processes and phenomena due to their nanoscale size [1].

Nanomaterial refers to a material with a crystalline or amorphous particulate structure on a nanoscale. The specificity of the size and structure of nanomaterials makes them have special properties not found in many traditional materials. The development of new nanostructured materials usually leads to a major advance in the field of high technology, of both academic and industry interest [2].

Among the nanomaterials widely used in various industrial sectors is Carbon Black (CB), which is the nanocarbon produced on a larger scale and commercially cheaper. The CB are aggregated of seheroidal nanoparticles, typically 10 to 100 nm in diameter and represent a very wide family of materials, with many commercial products available on the market, with a diverse variety of technological specifications and performance requirements. Its applications include the areas of nanotechnology, oil exploration and reservoir characterization, as well as use in medicine, catalysis and environmental remediation [3].

CB has low dispersion in polar solvents and, as a strategy to adjust the interactions of the particle surface with the medium and improve its dispersability and compatibility, can be covalently bonded to polymers. In this sense, the CB was functionalized with ethylenediamine (EDA) to produce the nanoparticle called CB-EDA[4]. It is believed that the toxicity of nanoparticles is determined by their surface reactivity, influenced by their chemical composition, physical properties and chemical surface. Thus, this modification with EDA can influence the biological activity of the nanoparticle, due to modified physical-chemical properties, resulting in potential CB-EDA toxicity and altering its biological effects[5].

Growing evidence links nanomaterials to adverse biological outcomes and, due to the variety of applications and potential human exposures to nanoparticles, it is important to assess their toxicity to determine the risk of workers and consumers. It is crucial to understand the underlying mechanisms of its toxicity, as the observation of similar effects after different exposures of nanomaterials does not reflect intracellular processing and interactions in similar organelles. A complete understanding of the mechanisms is necessary not only for predicting potential toxicological impacts, but also for the development of safer applications, modulating the physical-chemical characteristics [6].

Studies evaluating the toxic potential of CB-EDA are still scarce, which makes it extremely important that the performance of this nanoparticle be explored. The tests may include *in vitro/in vivo* analyses, seeking to understand how oxidative pathways, represented by the production of reactive oxygen species (ROS) and reactive nitrogen species (NOS), are related to the synthesis and release of pro-inflammatory molecules(7)].

In vitro and *in vivo* studies have demonstrated the ability of CB nanoparticles to induce toxicity to the respiratory system, but most of them did not address the molecular pathways of toxicity[6].

In view of this, it is necessary to evaluate the cytotoxic effects of the modified nanoparticle CB-EDA in biological models and it is expected that it will present low harmful effects in cell culture models, based on its surface modification and physical-chemical properties. Therefore, this study aims to evaluate the cytotoxic profile of CB-EDA Carbon in the AML-12 lineage (murine hepatocytes), in acute exposure for 24 h. For this, the potential cell damage was investigated by oxidative and inflammatory profile analysis in these cells, in addition to the metabolic pathways involved in this process.

MATERIAL AND METHODS

Nanoparticles

The CB-EDA nanoparticle was ceded by the petroleum industry Centro de Pesquisa Leopoldo Américo Miguez de Mello CENPES/Petrobrás, Rio de Janeiro, RJ, Brazil. PETROBRAS.

Cell Line AML-12

The hepatocytes lineage (AML-12) derived from liver tissue of *Mus musculus* was acquired in the cell bank of Rio de Janeiro (BCJ) code:0354. The cultivation occurred in bottles containing DMEM F12 (Dulbecco's Modified Eagle's Medium, Sigma) with 10% fetal bovine serum (LGC Biotechnology), at 37 ºC and with 5% CO2. A protocol was established with 3 independent experiments and in triplicates with the following concentrations of the black carbon nanoparticle modified with ethylenediamine (NP CB-EDA): 1, 50, 250, 500 and 1000 µg/mL for 24 hours. The selection criterion was to use concentrations that proved to be more toxic and nontoxic among those initially tested (1, 10, 50, 100, 250, 500, 750 e 1000 mg/ml) for 12,24,48 and 72hours. the concentrations chosen and hours was selected due to the decrease in cell viability.

Cell Viability by MTT Method

The colorimetric method of Tetrazolium 3 - (4.5 - dimethyltiltiazol - 2 - il) 2.5 - diphenylbromide (MTT) was performed (SIGMA-ALDRICH, USA) (MOSMANN, 1983) to evaluate the integrity of mitochondrial function by measuring the formation of formazan crystals by mitochondrial enzymes. The higher the production of these crystals, the greater the cell viability. In a microplate of 96 flat bottom wells, with treatment for cultivation and with lid (Corning® Costar 3527), 1×104 cells per well were inoculated. After 24 hours of support, THE CB-EDA NP was added. After the exposure times of 24 hours, the wells were washed 2 times with PBS 1X (Saline phosphate buffer: 8 g NaCl, 0.2 g KCl, 1.15 g Na2HPO4, 0.2 g KH2PO4 and 1 L distilled water) e 200 µl of MTT solution at 0.5 mg/mL in PBS 1X plus incomplete and phenol-without DMEM F12 medium (1:5) were added. The reaction occurred for 4 hours at 37 ºC and 5% CO2. White controls containing only MTT

solution were performed. Then, the reagent solution was removed and 100 µl of the DMSO diluent were added per well, followed by the absorption reading at 540 nm in spectrophotometer (Thermo Scientific ™ Multiskan ™ GO Microplate Spectrophotometer). Cell death control (CTRL+) was done with extra5% enzymatic detergent. The percentage of cytotoxicity occurred by comparing the data obtained with the CTRL group according to the equation.

$$\% \text{ cytotoxicity} = \frac{\text{Experimental group} \times 100}{\text{Control Group}}$$

Viability by Neutral Red Method

The neutral red assay is based on dye accumulation in liposometropic membranes. The more viable cells, the greater the diffusion of the dye through the membrane (REPETTO; DEL PESO; ZURITA, 2008). In a microplate of 96 flat bottom wells, with treatment for cultivation and with lid (Corning® Costar 3527), 1×10^4 cells per well were inoculated. After 24 hours the NP CB-EDA have been added. After the exposure time of 24 hours, the wells were washed 2 times with PBS 1X and 100 µL of dye at 30 µg/mL in DMEM F12 medium without phenol supplemented with 1% of SBF were added. The reaction occurred for 2 hours at 37 ºC and 5% CO2. Then the reagent solution was removed and 200 µL of the Acetic Acid diluent 1% and Ethanol 50% were added per well, followed by the absorption reading at 570 nm in spectrophotometer (Thermo Scientific ™ Multiskan ™ GO Microplate Spectrophotometer).

Cellular Morphology by Optical Microscopy

The cellular morphology of the AML-12 after exposure to nanoparticles was observed by optical microscopy. In microplate of 96 flat bottom wells, with treatment for cultivation and with lid (Corning® Costar 3527), 1×10^4 cells per well were inoculated. After 24 hours of support, THE CB-EDA NP was added. After the exposure time of 24 hours, the wells were observed in optical microscope Axiovert 40 CFL (Zeiss), with objective lens 10X, whose images were captured with the camera coupled model LOD-3000 (Bio Focus) and analyzed by future WINJOETM version 2.0 software in final resolution of 100X.

Reactive Nitrogen Species (NOS) Production by Griess Reaction

The production of NOS analyzed by means of the Griess reaction, which assing the production of nitrite ion (NO2-) (GREEN et al., 1982; SALTZMAN, 1954). In microplate of 96 flat bottom wells, with treatment for cultivation and with lid (Corning® Costar 3527), 1×10^4 cells per well were inoculated. After 24 hours of support, THE CB-EDA NP was added. After the 24-hour exposure time, 50 µL of the antinatant was collected and added to a new plate followed by the addition of 50 µL of griess solution (mixture 1: 1 of solution A [sulfanilamide 1% in phosphoric acid 5%] and solution B [0.1% n-1-naphtiletilenediamine dicloridra]) for 15 minutes at room temperature. The absorbance was read at 540 nm in spectrophotometer (Thermo Scientific ™ Multiskan ™ GO Microplate Spectrophotometer). The nitrite concentration in the sobrendante was quantified from a standard curve with known concentrations of nitrite in mM described in the kit.

Production of Reactive Oxygen Species (ROS)

The production of ROS was measured using the dcfh-da fluorescent probe (Dichlorodihydrofluorescein Diacetate 2′-7′-Dilorodihydrofluorescein) (Sigma-Aldrich) (WAN, MYUNG, LAU,1993). In a plate of 96 wells, $1x10^4$ cells/well were cultivated. After 24 hours for cell support, CONCENTRATIONS of BCP-EDA NP were applied. After exposure (24 hours), DCFH-DA probe was diluted in DMEM F12 medium and applied to each well (200µL) for 30 minutes. The wells were washed with PBS 1X and fluorescence emission was read at 485-530 nm in Spectra MAX, i3 plate spectrophotometer ® (Molecular Devices). The values obtained were transformed into percentages using the same formula previously mentioned in the MTT test.

Detection of IL-6 and TNF

For the measurement of il-6 and TNF cytokines (Kit OptEIATM, BDBiosciences) direct ELISA was performed following the protocol described below. For this procedure, high affinity microtitration plates with 96 wells (Corning® Costar 3590) were also used. Between each step the plates were washed with 300 µL/well of blocking solution (PBS 1X + + Tween 20 to 0.05%). After sensitization of the plates with 100 µL/well of specific capture antibody in carbonate buffer, there was incubation for 16 hours at 20 ºC. Then, the blocking reaction was performed with 200 µL/10% milk well, followed by incubation for 1 hour at room temperature. It was then performed the application of 50 µL of the samples and the titration curve of the cytokine patterns, with incubation 2 hours also at room temperature. The capture antibody conjugated with the enzyme peroxidase was added in the volume of 100 µL/well under light for 1 hour and 30 minutes. Then 100 µL/well of the TMB substrate was applied and the plates were incubated, still under light, from 15 to 30 minutes approximately. Then, the reaction was blocked with the addition of 50 µL/well of 2N sulfuric acid and the plate reading was performed in the plate spectrophotometer (Thermo Scientific ™ Multiskan ™ GO Microplate Spectrophotometer), at a wavelength of 450 nm. The concentrations were calculated from the titration curve of cytokine patterns and the final concentrations expressed in pg/mL.

Statistical Analysis

The data obtained in this study were expressed in ± sD and analyzed using GraphPad Prism 7.0 (San Diego, California, USA). The entire study was carried out in at least three biological triplicates and in three independent experiments. The discrepant data were identified by Grubbs analysis, followed by the Shapiro-Wilk test to verify the parametric or

nonparametric nature of the data. For this, the ANOVA test (variance analysis) was applied to parametric data and the tukey multiple comparation scan (the results were presented in mean and standard deviation). For nonparametric data, the Kruskal-Wallis test and dunn's multiple comparison post-test were used. Statistical significance was established at $p < 0.05$.

RESULTS

To define the concentrations of NP CB-EDA that would be exposed to AML-12 hepatocytes cells to be evaluated in the study, a preliminary cell viability test was performed using the MTT method. The test consisted of serial dilution of THE CB-EDA NP in 1, 10, 50, 250, 500, 750 and 1000 μg/mL and exposed to cells for 12, 24, 48 and 72 hours, represented in Figure 1. Data analysis indicated that in the 12-hour period there was no significant decrease in the cellular viability of AML-12 hepatocytes due to exposure to CB-EDA NP (Figure 1A). In 24 hours, there was a significant decrease in concentrations 100, 250, 500, 750 and 1000 μg/mL when compared with the control group - (Figure 1B). In 48 and 72 hours there was a significant decrease in cell viability at the same concentrations 250, 500, 750 and 1000 μg/mL when compared with the control group - (Figure 1C and 1D). These data served as the basis for the construction of ec50 of a graduated dose-response curve, represented by the concentration of the compound for which 50% of the expected effect is observed. In this case, at 12h the EC50 was 255.3; in 24h was 110; in 48h it was 269.3 and in 72h it was 279.3 (figure 1E-1H). In view of these results, the exposure time chosen to continue the tests was 24 hours, with concentrations of 1, 50, 250, 500 and 1000 μg/mL.

Figure 2 shows the results of the cytotoxicity profile by the neutral red dye methodology, at the concentrations and times defined. It can be observed that in 24 hours there was a significant reduction in the number of viable cells in the AML-12 scan at concentrations of 250, 500 and 1000 μg/mL when compared to the control group -.

Figure 3 shows, by qualitative data, the cellular morphology of AML-12 hepatocytes after 24 hours of exposure to NP CB-EDA, at different concentrations of 1, 50, 250, 500 and 1000 μg/mL and control group -. It is possible to observe a decrease in the size and alteration of the shape of the cells in the groups that were exposed to concentrations of 250, 500 and 1000 μg/mL, when compared to the control group -. The quantification of the production of reactive nitrogen species (NOS) and reactive oxygen species (ROS) for oxidative stress analysis were performed in the aml-12 murine hepatocyte lineage in the 24-hour period, at different concentrations of THE BC-EDA NP, as observed in Figure 4.

To produce NOS, observed in Figure 4A, only at the concentration of 1000 μg/mL there was a significant increase when compared to the control group -. In ROS production, observed in Figure 4B, there was a significant increase in concentrations of 50, 250, 500 and 1000 μg/mL when compared to the control group -.

The production of IL-6 and TNF cytokines was analyzed after 24 hours of exposure of the AML-12 murine hepatocytes to The NP CB-EDA, as shown in Figure 5. There was no significant variation in IL-6 production in the exposed groups when compared to the control group - (Figure 5A). In the production of TNF, a significant increase was observed in the group exposed to 1000 μg/mL of NP CB-EDA when compared to the control group - (Figure 5B).

DISCUSSION

In vitro models were developed in cytotoxicity studies to eliminate some variables, to obtain better control over the experimental conditions by the researchers. Several studies have evaluated the harmful effects of carbon nanoparticles in different cell types, including macrophages, lymphocytes and fibroblasts [8]. In addition to these cells, hepatocytes are also widely used in cytotoxicity evaluation tests. Primary hepatocytes are differentiated cells capable of reproducing the liver response *in vitro* and are currently considered a valuable tool to determine the metabolism of compounds and assess the risk of hepatotoxicity. As an alternative to the use of cultured primary hepatocytes, the use of liver cell lines has been established, which facilitates the development of tests [9]. Toxicity studies using hepatocytes are of fundamental importance, since hepatotoxicity can predict conditions such as intoxication, cancer and others, with similar potential results *in vivo* [10].

When enteringcontact with the AML-12 cell line, CB-EDA nanoparticles trigger a mechanism that results in decreased cell viability at the highest concentrations tested when compared to the control group. These data corroborate other studies that used the ultrathin Carbon Black nanoparticle exposed to murine hepatocytes, whose greatest reductions in cell viability also occurred as nanoparticle concentrations were higher [11, 12].

This reduction in cell viability may be correlated with oxidative stress pathways by ros and NOS production, which, when increased, result in cellular disability of redox control [13]. Reactive oxygen species and reactive nitrogen species are produced in normal hepatocytes and are critical to normal physiological processes including oxidative respiration, growth, regeneration, apoptosis and microsomal defense. When oxidation product levels exceed the capacity of normal antioxidant systems, oxidative stress occurs. This type of stress, in the form of ROS and NOS, can be harmful to all liver cells, including hepatocytes [14].

The main targets of oxidative stress are proteins, lipids and DNA/RNA, and modifications to these molecules may increase the chances of mutagenesis. Overproduction of ROS and NOS, notified for an extended period, can cause damage to cell structure and functions and may induce somatic mutations and preneoplastic and neoplastic transformations. In fact, this process can cause irreversible damage to cells, resulting in cell death by necrotic and apoptotic mechanisms [15]. In response to inflammatory stimuli, the increase in

NOS in hepatocytes is considered a short-lived cell messenger, which causes its production to be detected early [16].

Niranjan e Thakur (2017), in their studies, address oxidative stress and inflammation resulting from exposure of CB particles in *in vivo* experiments, highlighting the presence of DNA damage mediated by exacerbated ROS production [17]. The increase in ROS production was discussed by Requardt et al (2019), in *in vitro* studies based on the cellular exposure of the HepG2 strain to carbon nanotubes [18]. The increase in NOS production is also a mechanism discussed in the literature in the context of nanopoartículas, increasingly present in daily life, and detected by authors in studies such as: LA-9 cells exposed to NP CB-EDA [7] exposed to NP ONCT-TEPA [19].

Several inflammatory stimuli such as excessive ROS/NOS produced in the process of oxidative metabolism, or resulting from chemical compounds, have been reported as initiators of inflammatory process, resulting in the synthesis and secretion of pro-inflammatory cytokines. The activation of nuclear-kappa Factor B and the production of tumor necrosis factor alpha (TNF-α) were, for example, documented as playing a critical role in the inflammatory process [15]. In this sense, the production of TNF induced by exposure of AML-12 to CB-EDA was investigated, with the expected increase in the production of this cytokine in response to the inflammatory stimulus exerted in the cell by the nanoparticle.

TNF plays an important role in hepatocytes injury and cell death in various pathophysiological states. The TNF-induced cell death signaling pathway involves binding the linker to the TNF type I receptor, the recruitment of a series of intracellular proteins, and the activation of caspases whose proteolytic activities mediate apoptotic cell death, in addition to the independent pathways of caspase and necrosis. Considerable interest focused on the role of antioxidants in regulating hepatocytes sensitization to TNF and induced apoptosis due to the presumed role of oxidative stress in this death [20].

Estudos de Jiang et al. (2020) investigated the toxicity of carboxylated CB in human bronchial epithelial cells (BEAS-2B). Their results showed that the nanoparticle significantly increased the production of IL-6 and TNF-α in cells, in addition to identifying the correlation between ROS generation and the increased production of these inflammatory cytokines induced by carboxylated CB [21].

CONCLUSION

In view of the studies presented, it is observed that generation of ROS/NOS in the induction of oxidative stress, as well as the inflammatory profile of cytokines, are used as good indicators to evaluate the toxicity induced by nanoparticles, which compromise cell viability. According to the analyses of this study, THE CB-EDA NP has a cytotoxicity profile in the AML-12 cell line, evidenced by the induction of oxidative stress and secretion of inflammatory cytokines, with consequent decrease in cell viability. Thus, it is suggested that this toxicity be further explored in future studies, emphasizing the importance of elucidating mechanisms involved in cell damage and considering the increasing human and environmental exposure to nanoparticles.

ACKNOWLEDGEMENTS

The authors would like to thank Dra. Márcia Regina Cominetti (Departamento de Gerontologia, Universidade Federal de São Carlos, São Carlos, SP, Brazil) for availability of equipment. M.A. was supported by the Margarita Salas postdoctoral contract MGS/2021/21 (UP2021-021) financed by the European Union – Next Generation EU. The authors would also like to thank the financial aid from the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (2013/07296-2), the Financiadora de Estudos e Projetos – FINEP, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Financial Code 001) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq.

AUTHOR'S CONTRIBUTIONS

Study development: Krissia, Joice, Bruna.
Study design: Carlos, Fernanda.
Data analysis: Krissia, Joice, Bruna, Marcelo.
Preparation of the article: Krissia, Joice, Bruna, Luciana, Camila, Patricia, Marcelo.
Contribution of equipment and analysis: Elson, Marcelo.

FUNDING SOURCES

This study was supported by PETROBRAS/Project: Proc. No 2017/00010-7

STATEMENT OF ETHICS

The authors have no ethical conflicts to disclose.

DISCLOSURE STATEMENT

The authors have no conflicts of interest to declare.

REFERENCES

I. Nasrollahzadeh M, Sajadi SM, Sajjadi M, Issaabadi Z. An Introduction to Nanotechnology. 1st ed. Vol. 28, Interface Science and Technology. Elsevier Ltd.; 2019. 1–27 p.
II. Zhang L. Applications, challenges and development of nanomaterials and nanotechnology. J Chem Soc Pakistan. 2020;42(5):658–66.
III. Khodabakhshi S, Fulvio PF, Andreoli E. Carbon black reborn: Structure and chemistry for renewable energy harnessing. Carbon N Y [Internet]. 2020;162:604–49. Available from: https://doi.org/10.1016/j.carbon.2020.02.058
IV. Lima MCFS, Zaida do Amparo S, Ribeiro H, Soares AL, Viana MM, Seara LM, et al. Aqueous

suspensions of carbon black with ethylenediamine and polyacrylamide-modified surfaces: Applications for chemically enhanced oil recovery. Carbon N Y [Internet]. 2016;109:290–9. Available from: http://dx.doi.org/10.1016/j.carbon.2016.08.021

V. Lindner K, Ströbele M, Schlick S, Webering S, Jenckel A, Kopf J, et al. Biological effects of carbon black nanoparticles are changed by surface coating with polycyclic aromatic hydrocarbons. Part Fibre Toxicol. 2017;14(1):1–17.

VI. Boland S, Hussain S, Baeza-Squiban A. Carbon black and titanium dioxide nanoparticles induce distinct molecular mechanisms of toxicity. Wiley Interdiscip Rev Nanomedicine Nanobiotechnology. 2014;6(6):641–52.

VII. Rodolpho JM de A, Godoy KF de, Brassolatti P, Fragelli BD de L, Castro CA de, Assis M, et al. Apoptosis and oxidative stress triggered by carbon black nanoparticle in the LA-9 fibroblast. Cell Physiol Biochem. 2021;55(3):364–77.

VIII. Yuan X, Zhang X, Sun L, Wei Y, Wei X. Cellular Toxicity and Immunological Effects of Carbon-based Nanomaterials. Part Fibre Toxicol. 2019;16(1).

IX. Gomez-Lechon M, Donato M, Castell J, Jover R. Human Hepatocytes as a Tool for Studying Toxicity and Drug Metabolism. Curr Drug Metab. 2005;4(4):292–312.

X. O'Brien PJ, Chan K, Silber PM. Human and animal hepatocytes in vitro with extrapolation in vivo. Chem Biol Interact. 2004;150(1):97–114.

XI. Zhang R, Zhang X, Gao S, Liu R. Assessing the in vitro and in vivo toxicity of ultrafine carbon black to mouse liver. Sci Total Environ. 2019;655:1334–41.

XII. Li X, Chu S, Song Z, He F, Cui Z, Liu R. Discrepancy of apoptotic events in mouse hepatocytes and catalase performance: Size-dependent cellular and molecular toxicity of ultrafine carbon black. J Hazard Mater. 2022;421(June 2021):126781.

XIII. Antonucci S, Di Lisa F, Kaludercic N. Mitochondrial reactive oxygen species in physiology and disease. Cell Calcium. 2021;94(December 2020):102344.

XIV. Diesen DL, Kuo PC. Nitric oxide and redox regulation in the liver: Part II. Redox biology in pathologic hepatocytes and implications for intervention. J Surg Res. 2011;167(1):96–112.

XV. Hussain T, Tan B, Yin Y, Blachier F, Tossou MCB, Rahu N. Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? Oxid Med Cell Longev. 2016;2016.

XVI. Morgan ET, Skubic C, Lee C myung, Cokan KB, Rozman D. Regulation of cytochrome P450 enzyme activity and expression by nitric oxide in the context of inflammatory disease. Drug Metab Rev. 2020;52(4):455–71.

XVII. Niranjan R, Thakur AK. The toxicological mechanisms of environmental soot (black carbon) and carbon black: Focus on Oxidative stress and inflammatory pathways. Front Immunol. 2017;8(JUN):1–20.

XVIII. Requardt H, Braun A, Steinberg P, Hampel S, Hansen T. Surface defects reduce Carbon Nanotube toxicity in vitro. Toxicol Vitr. 2019;60(March):12–8.

XIX. de Godoy KF, de Almeida Rodolpho JM, Brassolatti P, de Lima Fragelli BD, de Castro CA, Assis M, et al. New Multi-Walled carbon nanotube of industrial interest induce cell death in murine fibroblast cells. Toxicol Mech Methods. 2021;31(7):517–30.

XX. Czaja MJ. Induction and regulation of hepatocyte apoptosis by oxidative stress. Antioxidants Redox Signal. 2002;4(5):759–67.

XXI. Jiang N, Wen H, Zhou M, Lei T, Shen J, Zhang D, et al. Low-dose combined exposure of carboxylated black carbon and heavy metal lead induced potentiation of oxidative stress, DNA damage, inflammation, and apoptosis in BEAS-2B cells. Ecotoxicol Environ Saf. 2020;206(September):111388.

Figure 1. Cell viability profile (mtt) of AML-12 hepatocytes in the face of NP CB-EDA exposure. Profile of cell viability in AML-12 murine hepatocytes cells when exposed to NP CB-EDA and definitions of concentrations and time to be applied in other methodologies. (A-D) MTT graphs at the 12–72-hour times of the concentrations exposed to NP CB-EDA when compared with the Control-group. (E-H) EC50 charts at the various times applied. * p< 0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs Control.

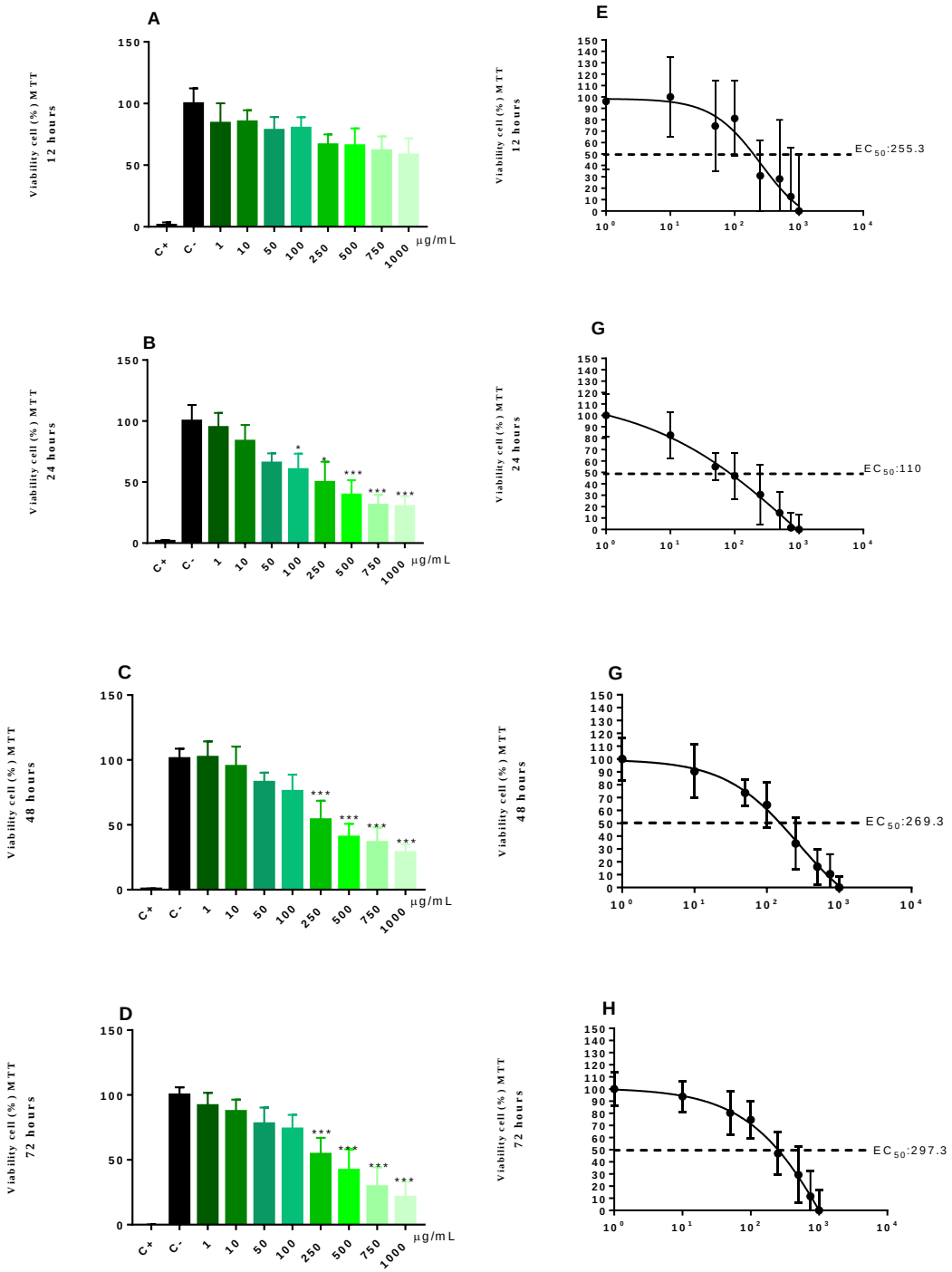
Figure 2. Profile of aml-12 cellular viability against NP CB-EDA exposure by neutral red dye method. Cell viability profile (neutral red) in AML-12 murine hepatocytes cells exposed to CB-EDA NP for 24 hours at concentrations 1, 50, 250, 500 and 1000 μg/mL and compared with the Control -group. * p< 0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs Control in 24 hours.

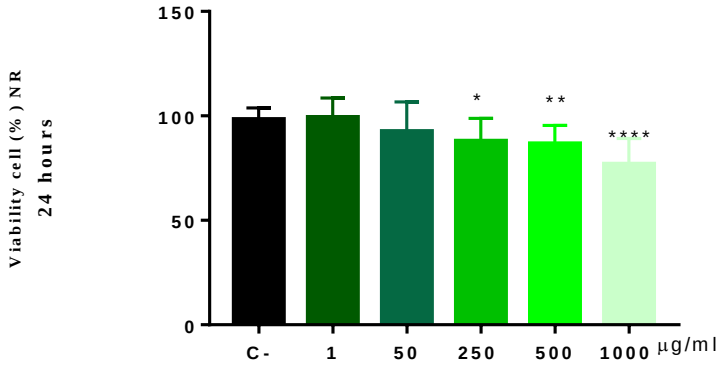
Figure 3. Cell morphology of the AML-12 lineage. Representative morphology by optical microscopy images with an increase of 100X of the groups exposed to NP CB-EDA at concentrations of 1, 50, 250, 500 and 1000 μg/mL and control group – in 24 hours. Qualitative data.

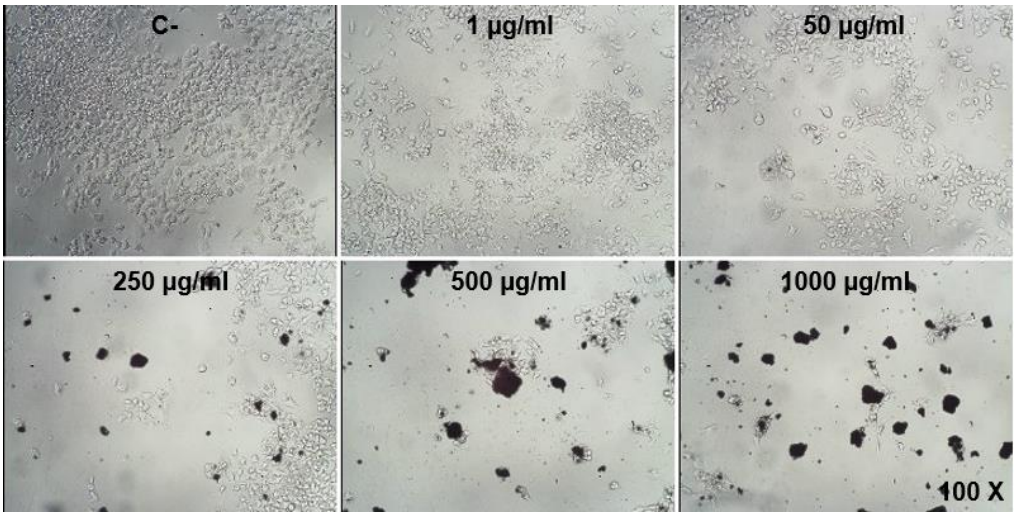
Figure 4. Oxidative stress pathways: production of reactive nitrogen and oxygen species (NOS and ROS). Oxidative stress pathways: (A) detection of NOS and (B) ROS detection. Evaluation in 24 hours of exposure to NP CB-EDA at different concentrations (1, 50, 250, 500 and 1000 µg/mL), compared with the Control-group. * p< 0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs Control in 24 hours.

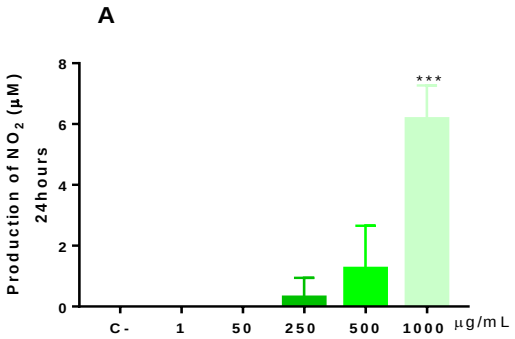
Figure 5. Cytokine levels in samples of aml-12 cells exposed to NP CB-EDA for 24 hours. Evaluation of IL-6 and TNF levels in the aml-12 cell overn in the control group and different concentrations of NP CB-EDA (1, 50, 250, 500 and 1000 µg/mL) exposed to cells. * p< 0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs Control in 24 hours

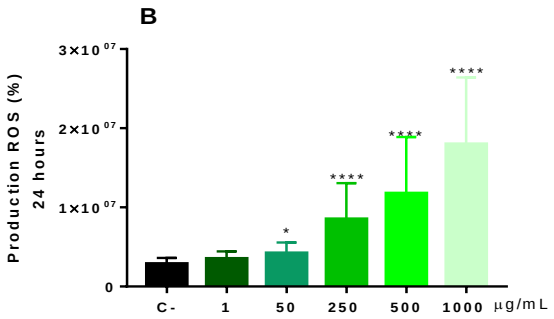
FIGURES 1 TO 5

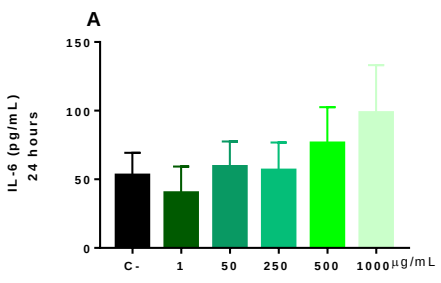


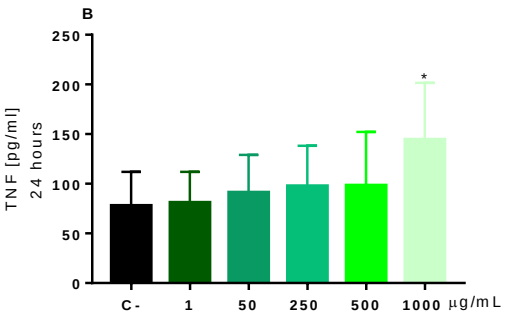

Viability cell (%) NR
24 hours
150
100
50
0
*
**
****
C-
1
50
250
500
1000
µg/ml


C-
1 µg/ml
50 µg/ml
250 µg/ml
500 µg/ml
1000 µg/ml
100 X


A
Production of NO2 (µM)
24hours
8
6
4
2
0
***
C-
1
50
250
500
1000
µg/mL


B
Production ROS (%)
24 hours
3x10 07
2x10 07
1x10 07
0
*
****
****
****
C-
1
50
250
500
1000
µg/mL


A
IL-6 (pg/mL)
24 hours
150
100
50
0
C-
1
50
250
500
1000µg/mL


B
TNF [pg/ml]
24 hours
250
200
150
100
50
0
*
C-
1
50
250
500
1000
µg/mL