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CYTOTOXICITY ASSESSMENT OF MULTI-WALLS CARBON NANOTUBES (MWCNT) CULTURE IN SECONDARY RAW 264.7 MACROPHAGES[1]

AVALIAÇÃO DA CITOTOXICIDADE DE NANOTUBOS DE CARBONO DE PAREDES MÚLTIPLAS (NTCPM) EM CULTURA SECUNDÁRIA DE MACRÓFAGOS RAW 264.7

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ABSTRACT

Nanotechnology has been developed rapidly in recent years due to its applications in different sectors such as electronics, energy, and the pharmaceutical industry (DURAN et al., 2006; ANNON, 2011). Among the most widely used nanomaterials, the use of carbon nanotubes (CNTs) stands out for their applications, such as drug carriers and biosensors and thus its biocompatibility and toxicity should be studied. In this study, the cytotoxicity of multiple wall carbon nanotubes (MWCNTs) in RAW 264.7 macrophages at concentrations of 1 and 5% of MWCNTs and 1 μg / ml of Escherichia coli lipopolysaccharide (LPS) was evaluated for 24 and 48 hours. Cytotoxicity was assessed by the release of lactate dehydrogenase (LDH), nitric oxide (NO) and gene expression of cytokines TNF-α and IL-1β. The results demonstrated that the MWCNTs at the concentrations that were used had no cytotoxic effect on the cells tested.

Keywords: nanotoxicology, nitric oxide, cells viability.

RESUMO

A nanotecnologia tem se desenvolvido rapidamente nos últimos anos, devido as suas aplicações em diferentes setores como, eletrônica, energia, e na indústria farmacêutica (DURAN et al., 2006; ANNON, 2011). Dentre os nanomateriais mais utilizados, destaca-se o uso dos nanotubos de carbono (NTC) por suas aplicações, como transportadores de drogas e biossensores e, assim, sua biocompatibilidade e toxicidade devem ser estudadas. Neste estudo, foi avaliada a citotoxicidade r nanotubos de carbono de paredes múltiplas (NTCPM) em macrófagos RAW 264.7 nas concentrações de 1 e 5 % de NTCPM e 1 μg/ml de lipopolissacarideo de Escherichia coli (LPS) por 24 e 48 horas. A citotoxicidade foi avaliada por liberação da enzima Lactato desidrogenase (LDH), pela dosagem de óxido nitríco (NO) e expressão gênica das citocinas TNF-α e IL-1β. Os resultados demonstraram que os NTCPM nas concentrações utilizadas não apresentaram efeito citotóxico nas células testadas.

Palavras-chave: *nanotoxicologia, óxido nítrico, viabilidade celular.*

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INTRODUCTION

Nanotechnology has been developed rapidly in recent years, seeking applications in different sectors, from electronics, energy, to the pharmaceutical industry (DURAN et al., 2006; ANNON, 2011). Nanomaterials have different chemical, physical, physicochemical and behavior properties regarding larger-scale materials (ANNON, 2011). With these materials it is possible to manipulate atoms and molecules of any living being and any object (MEDEIROS; MATOSSO, 2006).

Among nanomaterials, carbon nanotubes (CNTs) belong to a new class of materials with extraordinary mechanical and electronic properties, which has aroused the interest of a large number of researchers (LIU et al., 2008). CNT can be classified into two types: single walled carbon nanotubes (SWCNT) which are formed by a graphene sheet, which is rolled together, thus forming a cylinder, which may have different shapes like *armchair*, *zig zag* or *chiral*. These may have diameters between 0.4 to 2.0 nm and lengths in 20-1000 nm. The second form is the multiple wall (MWCNT), which have multiple sheets of graphene, concentrically coiled and have diameters in the order of 1.4 -100 nm and lengths of 1 to several micrometers, forming a rigid structure (HERBEST, 2004).

The biocompatibility of CNTs is a challenge in biomedicine (SMART et al., 2006). It is necessary to understand the biological compatibility of materials that are used successfully in biomedical implants, biosensors, drug delivery vehicle and vaccine (CUI et al., 2010). Several biomedical carbon compounds devices showed a great biocompatibility, besides great advantages in biomedical applications. However, when they are subjected to *in vitro* tests, the CNT can cause toxicity (CHLOPEK et al., 2006).

Studies show that CNTs biocompatibility with cells of the immune system depends primarily on the chemical compounds present on the surface of the nanomaterials. When they are functionalized CNTs can improve the biocompatibility as well as stability and solubility, thus reducing cytotoxicity in macrophages. However, the death of phagocytic cells was dependent on CNTs dose (KAGAN et al., 2005; POTER et al., 2009).

According to Sakamoto et al. (2009), after administration of CNTs in different exposure conditions, they can be captured by macrophages present in the administered region (OYEWUMI et al., 2010). However, when CNTs are provided with the biological system, nanomaterials may affect different intracellular structures, and also enable various enzymatic pathways, which results in different types of damage/cell injury (SHVEDOVA et al., 2012). Studies show that MWCNT can internalize into cells, overcoming the barriers to reach the cell nucleus. CNTs are not internalized when functionalized, they are found in lysosomes in macrophages and cores (MUKAI et al., 2009).

According to Sohaebuddin et al. (2010), both the size and the surface area of CNTs composition and the type of cell affected are determining factors for the development of cellular response,

in addition to the degree of toxicity and potential mechanisms. Such injuries caused by CNTs may contain macrophages involved in the phagocytosis process and are constantly encountered due to the mechanism of toxicity, inflammation and carcinogenesis (POLAND et al., 2008). Thus, leading to MWCNT inflammasome activation by caspase-1, causing the maturation of cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) (SCHRODER; TSCHOPP, 2010; LAMKANFI, 2011). The aim of this study was to evaluate the cytotoxicity caused by MWCNT in macrophages cell line RAW 264.7 grown *in vitro.*

MATERIAL AND METHODS

CARBON NANOTUBES WALLS-MULTIPLE

The non-functionalized MWCNT were purchased from Sigma-Aldrich Company, which have a size between 0.1 - 10 micrometres; inner diameter of 2-6 nm; outer diameter of 10-15 nm; and a purity higher than 90% depending on the manufacturer. These MWCNT were dispersed in DMEM culture at concentrations of 1 and 5 %.

CELL CULTURE

RAW macrophages 264.7 were cultured, the line was acquired from the Bank of cells of Rio de Janeiro, Brazil. The cell pellet was resuspended in 5 ml with culture medium DMEM / F-12 (L-glutamine, pyridoxine Hydrochloride, HEPES Buffer and Bicarbonate) medium supplemented with 10% fetal bovine serum (FBS), 100 U / ml penicillin and 100 µg / ml streptomycin. Cell viability was assessed by trypan blue method, which was used a volume of $5x10^5$ cells/mL. The cells were grown in culture bottles with complete DMEM medium in an atmosphere of 5% CO_2 at 37 °C and were maintained in culture until reaching a confluence of approximately 80% (AMARAL, 2004).

After adherence, cells were treated with 0.05% trypsin-EDTA to promote detachment of cells from the plates, cells were washed twice with PBS solution (Phosphate Buffered Saline) and transferred to 24 pits plates for achievement of the following treatments: a) only culture medium supplemented as the experiment control group; b) hydrogen peroxide at a concentration of 100 mM as a positive control for the experiment; c) MWCNT at a concentration of 1 %; d) MWCNT at a concentration of 5 % as a test; e) Lipopolysaccharide from *Escherichia coli* (Sigma-Aldrich) at a concentration of 1 %. The treatments were carried out for periods of 24 and 48 hours and plates were maintained at an atmosphere of 5% CO_2 at 37 °C.

QUANTIFICATION OF NITRIC OXIDE

The concentration of nitric oxide (NO) was determined by colorimetric assay using the Greiss method (GREEN et al., 1982). On a plate of 96 flat bottom pits, 50 µL of Greiss reagent (0.1% sulfanilamide, N-naphthyl-ethylenediamine 0.1% and 2.5% phosphoric acid) and 50 µL of cell supernatant treated with 1 and 5 % of MWCNT were added. After approximately 10 minutes at room temperature, the absorbance was read on microplate reader (Termoplate®) at a wavelength of 570 nm and compared to a standard curve of sodium nitrite prepared for the experiment. Data were expressed as µmols.

DETERMINATION OF LACTATE DEHYDROGENASE

The dosage of the enzyme lactate dehydrogenase (LDH) was conducted to assess the cytotoxicity caused by MWCNT in RAW 264.7 macrophages. LDH assay is performed by lactate dehydrogenase that is a cytoplasmic enzyme, which is used as a marker for cell damage *in vitro*. The enzymatic activity is stable in cell culture medium, the enzyme can be measured after leaving those cells with compromised membranes (non-viable cells) (KORZENIEWSKI; CALLEWAERT, 1983). It belongs to the class of oxi-reductases responsible for catalyzing and reversible oxidation of lactate to pyruvate, this process occurs in the presence of coenzyme NAD^+ which has a donor of paper or hydrogen acceptor (MOTTA, 2009).

The manufacturer's protocol (Labtest) was followed to perform LDH dosage in the cell culture. In each test tube, 1 ml of working reagent was placed, which had been kept in water bath at 37 °C for 1 minute. In thermal bucket, it was added 20 µL of cell supernatant and the working reagent at 37 °C for 1 minute. The reading of absorbance was determined by a Shimadzu spectrophotometer (UV-1650PC) at a wavelength of 340 nm.

RNA EXTRACTION

The procedure for RNA extraction was performed according to the manufacturer Life Technologies, without any changes. After the extraction of the RNAs, DNase treatment was performed and, for this, 2 µL of RNA from each sample was transferred to a new eppendorf, and 1 µL of DNase was added, 1 µL of the DNase buffer, 10 µL of the QSP nuclease free water. Then, the samples were incubated for 15 min at RT and added 1µL of 25 mM EDTA and incubated for 10 minutes at 65 °C in thermocycler Sxift Maxi (ESCO).

REVERSE TRANSCRIPTASE

Sigma-Aldrich protocol was used for the extraction of complementary DNA (cDNA). In the first phase of the cDNA synthesis reaction, the RNA was linearized by adjusting the Sxift Maxi thermal cycler (ESCO), at 70 ° C for 50 minutes, using the mixture I, consisting of 1 μL dNTP, 1 ML of oligo (dT18), 1 μL of extracted RNA and 10 μL of QSP Nuclease-free water.

In the second stage of the reaction, the thermocycler Sxift Maxi (ESCO) was adjusted to a temperature of 37 °C for 50 minutes and added to the mix I, II consisting of 2 μL reverse transcriptase buffer, 1 μL enzyme reverse transcriptase, 0.5 μL RNase inhibitor and 6.5 μL nuclease-free water.

For cDNA synthesis, followed by an incubation program at room temperature for 10 minutes and then incubated in a thermocycler Sxift Maxi (ESCO) for 50 minutes at 30 °C. The reaction tube was maintained at 94 °C for 10 minutes to denature the room temperature.

AMPLIFICATION BY CHAIN REACTION OF CONVENTIONAL POLYMERASE-PCR

Expression of the genes for the cytokines TNF-α and IL-β was evaluated by polymerase chain reaction using reverse transcriptase by conventional PCR of macrophages incubated with MWCNT and compared to control cells.

The pairs of primers (Ludwig) used in the PCR reaction are described in table 1. β-actin was used as an internal control. B actin was chosen by its constitutive expression pattern. The volumes of the reagents used for the run were the same, as shown in table 2. The program for conducting the PCR was at 95 ° C for 2 minutes for the denaturation of the sample; 94 ° C for 30 seconds for denaturation; 60 ° C for 50 seconds, for the annealing of the primers; 72 ° C for 1 minute, for elongation; 72 ° C for 5 minutes, for final elongation. The programming was 40 cycles in the Sxift Maxi thermal cycler (ESCO).

Table 1 - Sequences of primer pairs of forward (F) and reverse (R) used in a conventional PCR reaction.

PRIMERS F and R	SEQUENCE OF PRIMERS	SIZE OF BASIC COUPLE PRIMERS
TNF-α (F)	AGACCCTCACACTCAGATCATCTTC	148 pb
TNF-α (R)	TTGCTACGACGTGGGCTACA	
IL-1β (F)	CTGTGTCTTTTCCCGTGGACC	200 pb
IL-1β (R)	CAGCTCATATGGGTCCGACA	
β ACTIN (F)	AGAGGGAAATCGTGGCGTGAC	97 pb
β ACTIN (R)	ACGGCCAGGTCATCACTATTG	

Table 2 - Volume of reaction components for PCR.

REAGENTS	1x	Mix 4x
Buffer 10x (Taq)	2.5 µL	10µL
dNTPs 10 mM	2 µL	8 µL
$MgCl_2$ 2,0 mM	1 µL	4 µL
Primer (F)	1 µL	4 µL
Primer (R)	1 µL	4 µL
Water Q.S.P.	25 µL	100 µL

STATISTICAL ANALYSIS

Results were expressed as average and standard deviation. Statistical analysis was performed using analysis of variance (ANOVA), and the values were considered significant when p <0.001, 0.01 and 0.05. Data were analyzed using the Statistical Package for Social Science (SPSS) and Graph Pad Prism 5.

RESULTS AND DISCUSSION

CELL CULTURE

In order to standardize the macrophage cell culture of the RAW 264.7 secondary strain treated with MWCNT. It was possible to observe that the cells were able to internalize the MWCNT at a concentration of 1 mg / mL. However, we chose to use only the lowest MWCNT concentration, since there was no significant difference in the biochemical tests performed between the concentrations of 1 and 5 mg / mL.

Macrophages capture CNTs and pathogens that have larger sizes than 0.5 µM to try to eliminate them from the body mainly by the process of phagocytosis (ADEREM; UNDERHILL, 1999; HIROTA; TEREDA, 2012). Donaldson et al. (2010) observed that the longer nanotubes (15-20 µM) tend to produce inflammation and fibrosis when they come into contact with the peritoneal cavity, as opposed to short angled or nanotubes that do not cause significant inflammation. Thus, it can be said that MWCNT used in this work, which have a length of 0.1 - 10 micrometres did not cause cytotoxicity as we observed in LDH cytotoxicity testing.

Figure 1 - Culture of secondary macrophages of strain RAW 264.7 after 24 hours with the treatments signaled by letters A to D. A) Control group with culture medium, in the final increase of 200X; B) Negative control group, treated with 100 mM of hydrogen peroxide, with a final increase of 200X; C) Group treated with 1 mg / mL of NTCPM, with final increase of 400X; D) Group stimulated with 1 µg / mL of LPS positive control, with increase of 200X. The arrows suggest the internalization / endocytosis of MWCNT by secondary macrophages.

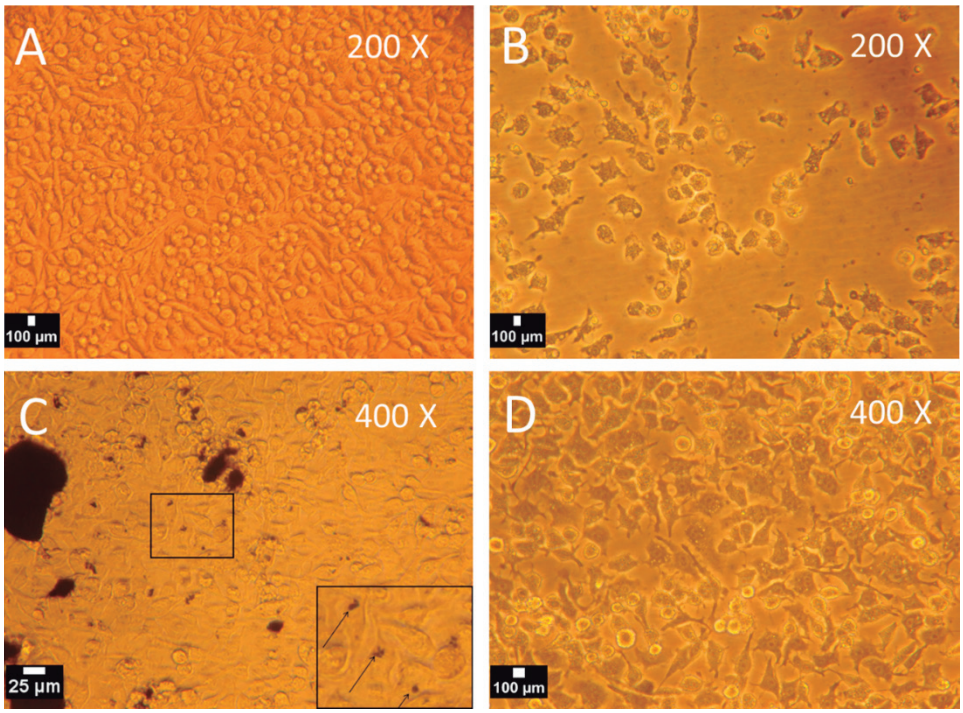
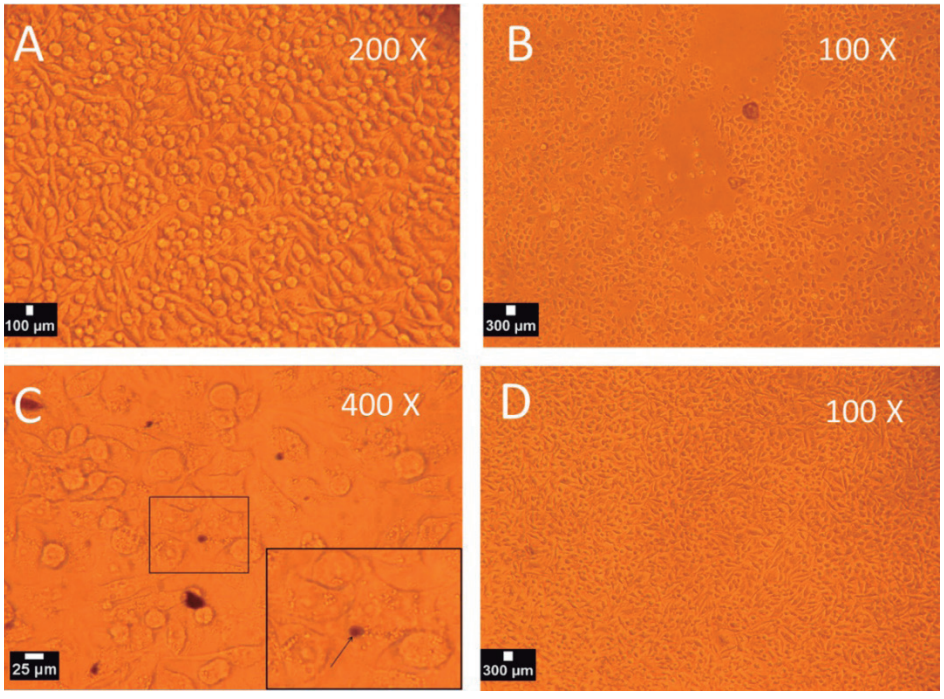


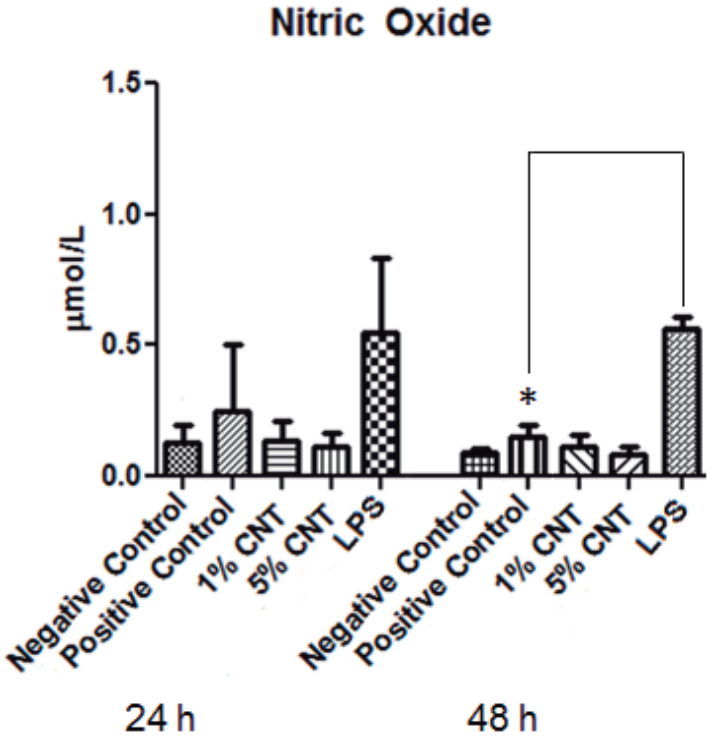
Figure 2 - Culture of secondary macrophages of the RAW 264.7 line after 48 hours with the treatments signaled by letters A to D. A) Control group with culture medium, in the final increase of 200X; B) Negative control group, treated with 100 mM of hydrogen peroxide, with final increase of 100X; C) Group treated with 1 mg / mL of MWCNT, with final increase of 400X; D) Group stimulated with 1 µg / mL of LPS positive control, with increase of 100X. The arrows suggest the internalization / endocytosis of MWCNT by secondary macrophages.



QUANTIFICATION OF NITRIC OXIDE

In this work, nitric oxide synthesis was used to evaluate NO release in RAW 264.7 macrophages, when they remained in contact in culture medium containing concentrations of 1 and 5 mg / mL MWCNT. NO determination was performed on supernatant of cell cultures for 24 and 48 hours in the treated cells. Cell cultures were performed with DMEM medium supplemented with 10% fetal bovine serum (FBS). Our results show that there was a significant NO release between the RAW 264.7 macrophages in relation to the negative control group and the positive control in the treatment period for 24 hours, as shown in figure 3.

Figure 3 - Production of NO in different concentrations MWCNT strain in the RAW 264.7 macrophage cultures. Each bar represents the average ± standard deviation of three independent experiments performed in triplicate. Where significant differences are represented by * between positive control and the negative control, within 48 hours. Statistical analysis was performed by analysis of variance (ANOVA) *p <0.05 compared to positive control.



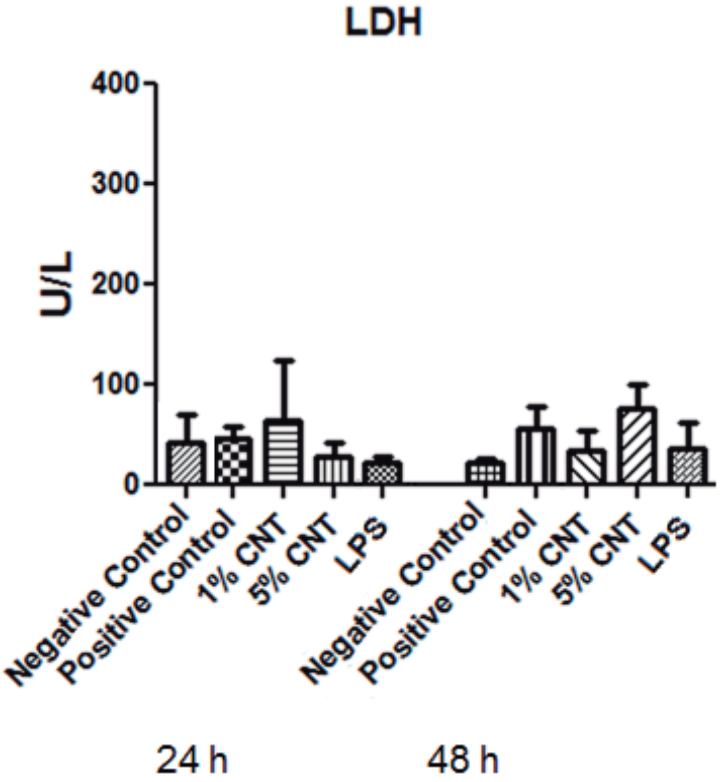
The results of this work demonstrated that the MWCNT were not able to stimulate an NO production in RAW 264.7 macrophages, which is in agreement with the findings obtained by optical microscopy, where we observed that no damage occurred from the macrophages that remained in contact with different concentrations of MWCNT.

DETERMINATION OF LACTATE DEHYDROGENASE

The effect of MWCNT after periods of 24 and 48 hours was evaluated by the damage to the cell membrane using LDH assay, where we used secondary line of RAW 264.7 macrophages. These

cells received the same treatments mentioned above. Our results show a significant release of LDH in the supernatant of RAW 264.7 cell line compared to the positive control group, and cells treated with 5 % MWCNT for periods of 24 and 48 hours, as shown in figure 4.

Figure 4 - Production LDH at different concentrations MWCNT in secondary cultures of RAW 264.7 macrophages. Each bar represents the average ± standard deviation of three independent experiments performed in triplicate. Statistical analysis was performed using analysis of variance (ANOVA), which showed no statistical difference.



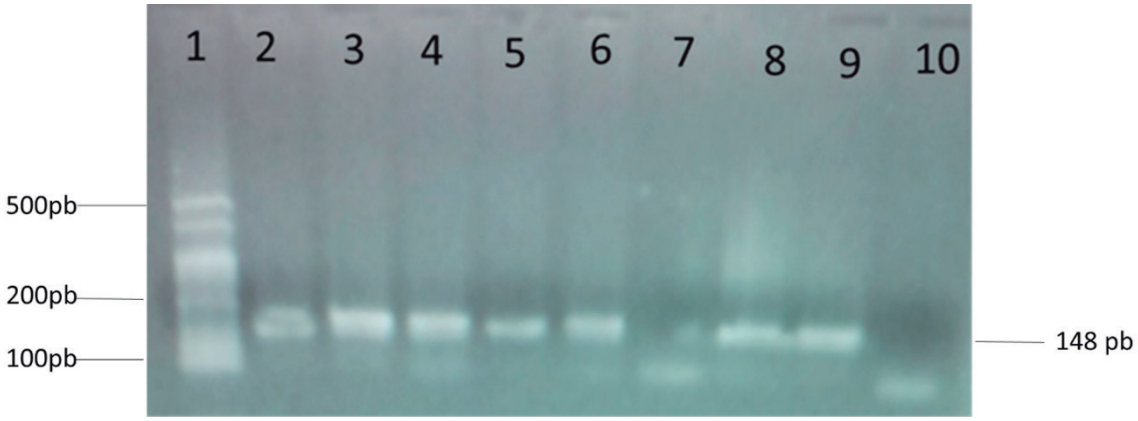
GENE EXPRESSION OF TNF-A AND IL-1B CYTOKINE IN RAW 264.7 MACROPHAGES STIMULATED WITH MWCNT

RNA extraction from RAW 264.7 cells, treated in the same manner as above, was performed using the commercial kit PURELINK MINI KIT AMBION. However, in the MWCNT group, only 1 mg / mL concentration was used, since in the previous biochemical tests, no significant difference was observed between the two treatments.

After checking the extraction of RNAs, treatment with DNAse and complement DNA synthesis (cDNA) was performed. PCR was then performed with the pair of primers for TFN-α as indicated in item 2.7. Figure 5 shows the 1% agarose gel following the PCR reaction, showing expected amplification in the size of approximately 148 bp for the TNF-α gene. In the gel, pit 1 corresponds to the molecular weight marker of 100 bp. Pits 1 through 5 represent the gene amplification for RAW 264.7 macrophages with culture medium, showing a basal expression of this cytokine, even without any stimulus. Pit 6 shows the expression of macrophages stimulated with 1 mg/mL

of MWCNT; pit 7 corresponds to the negative control, cells treated with 100 mM of hydrogen peroxide, where the RNA is not integrated and pits 8 and 9 present the macrophages stimulated with 1 µg / mL of LPS.

Figure 5 - Gene expression of TNF-α cytokine by RAW 264.7 macrophages incubated with MWCNT. 1) Molecular Marker 100 bp; 2-5) macrophages in culture medium - negative control of the experiment; 6) macrophages challenged with 1mg /mL MWCNT; 7) macrophages treated with 100 mM of hydrogen peroxide - positive control of the experiment; 8 and 9) macrophages treated with 1µg/mL of LPS.



In the same way, as for TNF-α, RNAs extraction, DNAse treatment, complementary DNA synthesis (cDNA), and PCR with the pair of primers for IL-1β were performed and the control with β actin as indicated in item 2.7. Figure 6 shows the 1.2% agarose gel following the PCR reaction, showing expected amplification at the size of approximately 200 bp for the IL-1β gene. A 50 bp molecular weight marker was used. Pits 1 to 4 represent the gene amplification for RAW 264.7 macrophages with culture medium, showing a basal expression of this cytokine, even without any stimulus. Pit 5 shows the expression of macrophages stimulated with 1 mg / mL of MWCNT; pit 6 corresponds to the negative control, cells treated with 100 mM of hydrogen peroxide where the RNA is not integrated and pits 7 and 8 present the macrophages stimulated with 1 µg / mL of LPS; And pit 9 corresponds to the test blank, composed only by a water sample, which is used as a standard control for the PCR analyzes.

As shown in figure 7, a 1.2% agarose gel was prepared after the PCR reaction, where it is possible to observe the amplification in the size of 97 bp for the β-actin gene, which was used as a control in the reaction. A 100 bp marker was used. Pit 1 represents the gene amplification of the RAW 264.7 macrophages with culture medium, and it is possible to notice that a basal expression of this gene occurred. The pit shows the macrophage gene expression stimulated with 1 mg/mL of MWCNT; pit 3 corresponds to the negative control where the cells were treated with 100 mM of hydrogen peroxide, where the RNA is not intact; pit 4 corresponds to the macrophages stimulated with 1µg / mL of LPS; and pit 5 corresponds to the blank test composed only of the sample with water, which is used as a standard control for the PCR analyzes.

Figure 6 - Gene expression of IL-1β cytokine by RAW 264.7 macrophages incubated with MWCNT. A molecular marker of 50 bp was used; 1-4) macrophages in culture medium - positive control of the experiment; 5) macrophages stimulated with 1mg/mL MWCNT; 6) macrophages treated with 100 mM of hydrogen peroxide - positive control of the experiment; 7 and 8) Macrophages treated with 1 μg/mL of LPS; 9) Control.

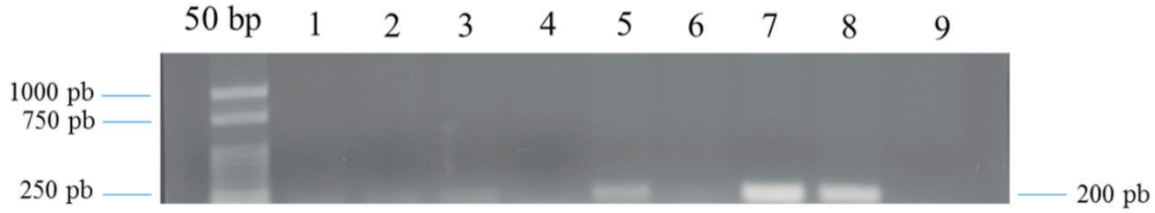
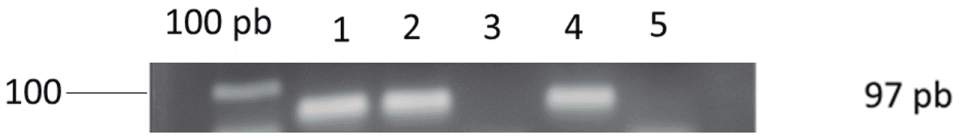


Figure 7 - Gene expression of β-actin by RAW 264.7 macrophages incubated with MWCNT. A molecular marker of 100 bp was used; 1) macrophages in culture medium - positive control of the experiment; 2) macrophages stimulated with 1mg/mL MWCNT; 3) macrophages treated with 100 mM of hydrogen peroxide - positive control of the experiment; 4) Macrophages treated with 1μg/mL LPS; 5) Control.



CONCLUSION

The results presented in this study have demonstrated that MWCNT with a variable length of 0.1 to 10 micrometres and with internal and external diameters between 2-6 nm and 10-15 nm respectively, when in contact with macrophages in concentrations of 1 and 5% were internalized in secondary line of RAW 264.7 macrophages.

The evaluation of MWCNT cytotoxicity was performed by the biochemical dosage of the LDH enzyme, since the other colorimetric assays are able to interact with the CNT, thus leading to false-positive and / or false-negative results. However, concentrations of 1 and 5% MWCNT when in contact with RAW 264.7 macrophages have showed a low release of LDH in the cell supernatant, indicating that these concentrations in MWCNT were not enough to cause significant toxicity. However, it is speculated that small pores in the cell membranes of macrophages may have been formed, thus enabling the release of NO molecules and pro-inflammatory cytokines such as IL-1β and TNF-α. Low production and release of NO indicates that the concentrations used in MWCNT may have led to a low intensity inflammatory response. This was also observed in tests using the molecular expression of IL-1β and TNF-α cytokines, which are released in the pro-inflammatory process. However, this inflammatory process caused by MWCNT in macrophages was not able to cause damage to the cell membrane, which was observed in optical microscopy and cytotoxicity tests.

The cell death mechanism of CNTs is not well known, however, when in contact with the biological system, CNTs are able to induce cell death by autophagy, which occurs through the forma-

tion of vacuoles or pyroptosis, which is mediated by releasing IL-1β. However, further studies will be necessary to understand exactly how cell death caused by MWCNT occurs. Whereas there is a growing interest in the medical and biological properties of CNTs, biomaterials containing carbon nanotubes are expected to be developed for clinical use. Therefore, studies on the effects of CNTs on the cells and tissues are extremely important for their *in vivo* use.

REFERENCES

ADEREM, A.; UNDERHILL, D. M. Mechanisms of phagocytosis in macrophages. **Annual Review of Immunology,** v. 17, p. 593-623,1999.

AMARAL, K. F. **Análise da citotoxicidade do EDTA e ácido cítrico aplicados em cultura de macrófagos peritoniais residentes**. 2004. 137f. Dissertação (Mestrado em Odontologia) - Faculdade de Odontologia, Universidade de São Paulo, São Paulo, 2004.

ANNON. Commission recommendetion on the definition of nanomaterial. **Official Journal of the European Union**, (L 275/38, 20 oct. 2011).

CHLOPEK, J. et al. *In vitro* studies os carbono nanotubes biocompatibility. **Carbon**, v. 44, n. 6, p. 1106-1111, 2006.

CUI, H. F. et al. Interfacing carbon nanotubes with living mammalian cells and cytotoxicity issues. **Chemical Research in Toxicology**, v, 23, n. **7**, p. 1131-1147, 2010.

DONALDSON, K. et al. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. **Particle and Fibre Toxicology**, v. 7, n. 5, p. 2587-2600, 2010.

DURAN, N.; MATTOSO, L. H. C.; MORAIS, P. C. **Nanotecnologia**. São Paulo: Art. Liber, 2006. p. 1-201

GREEN, L. C. et al. Analisys of Nitrate, Nitrite, and [^{15}N] Nitrate in Biological fluids. **Analytical Biochemistry,** v. 126, p. 131-138, 1982.

HERBEST, M. H. Tecnologia dos Nanotubos de Carbono: Tendências e Perspectivas de uma área multidisciplinar. **Revista Química Nova**, v. 27, n. 6, p. 986-992, 2004.

HIROTA, K.; TEREDA, H. Endocytosis of Particle Formulations by Macrophages and Its Application to Clinical Treatment. **Intech Open Science,** n. 16, p. 413-428, 2012.

KAGAN, V. E.; BAYIR, H.; SHEVDOVA, A. A. Nanomedicine and nanotoxicology: two sides of the same coin. **Nanomedicine**, v. 1, p. 313-316, 2005.

KORZENIEWSKI, C.; CALLEWAERT, D. M. An anzyme-release assay for natural cytotoxicity. **Journal Immunology Methods,** v. 64, p. 313-320, 1983.

LAMKANFI, M. Emerging inflammasome effector mechanisms. **Nature Review Immunology**, v. 11, n. 3, p. 213-220, 2011.

LIU, Z. et al. Drug Delivery with Carbon Nanotubes for *In vivo* Cancer Treatment. **Cancer Research**, v. 68, n. 16, p. 6652-6660, 2008.

MEDEIROS, E.; MATOSSO, L. Aplicações na nanotecnologia no agronegócio. In: **Nanotecnologia.** Arliber Editora Ltda, 13, p. 195-201, 2006.

MOTTA, V. T. Enzimas. **Bioquímica para Laboratório- Princípios e Interpretações.** 5. ed. Medbook, 2009. p. 108-110.

MUKAI, Y. et al. Structure-function relationship of tumor necrosis factor (TNF) and its receptor interaction based on 3D structural analysis of a fully active TNFR1-selective TNF mutant. **Journal of Molecular Biology**, v. 85, n. 4, p. 1221-1229. 2009.

OYEWUMI, M. O.; KUMAR, A.; CUI, Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. **Expert Review of Vaccines,** v. 9, p. 1095-1107, 2010.

POLAND, C. A. et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos--lik, pathogenicity in a pilot study. **Nature Nanotechnology**, n. 3, p. 423-428, 2008.

POTER, A. E. et al. Uptake of noncytotoxic acid-treated singlewalled carbon nanotubes into the cytoplasm of human macrophage cells. **ACS Nano**, v. 3, p. 1485-1492, 2009.

SAKAMOTO, Y. et al. Induction of mesothelioma by a single intrascrotal administration of multi-wall

carbon nanotube in intact male Fischer 344 rats. **Journal Toxicology Science***,* v. 34, n. 1, p. 65-67, 2009.

SCHRODER, K.; TSCHOPP, J. The inflammassomes. **Cell***,* v. 140, n. 6, p. 821-832, 2010.

SHVEDOVA, A. A. et al. Mechanisms of carbón nanotube-induced toxicity: focus on oxidative stress. **Toxicology and Applied Pharmacology**, v. 261, n. 2, p. 121-133, 2012.

SMART, S. K. et al. The biocompatibility of carbono nanotubes. **Carbon***,* v. 44, p. 1034-1047, 2006.

SOHAEBUDDIN, S. K. et al. Nanomaterial cytotoxicity is compositions, size, and cell type dependente. **Particle and Fibre Toxicology,** v. 7, n. 22, p. 2-17, 2010.