IN VITRO EVALUATION OF CYTOTOXIC, GENOTOXIC, MUTAGENIC AND CELL CYCLE EFFECT OF CARBON NANOTUBES

A THESIS

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By

CLAUDIA ESPINOSA

Directed by

Isabel Cristina Ortiz Trujillo. MSc, PhD.
Lina Marcela Hoyos Palacio. PhD.

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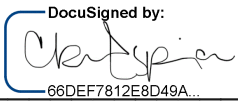
Declaration of Originality

Medellín, December 12th, 2016

I **Claudia Espinosa**

"Declare that this thesis has not been presented to apply to a title either in the same form or with variations in this or any other university" Art 82 Regimen Discente de Formación Avanzada.

Signature: 

To my father, Luis Eduardo Espinosa
For believing in me and making possible my academic career
I thank you with all my heart

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Abstract

Introduction: Nano-engineered materials such as carbon nanotubes (CNTs) have gained popularity due to their many characteristics which make them the ideal material for applications in biomedical areas. The use of CNTs in medical applications is growing but their interaction with the human body and the effects of those interactions are unknown or not investigated. The results of those interactions determine the clinical success of the use of CNTs in the medical field; therefore, it is necessary to evaluate the risk and possible secondary effects of CNTs on biological systems.

Objective: Evaluate the genotoxic and mutagenic activity of CNTs in human cells.

Methodology: The genotoxic and mutagenic activity of two types of CNTs was evaluated in human umbilical artery endothelial cells (HUAEC), HepG2, human lymphocytes, and *S. thyphimurium*. The effects produced by CNTs on the target cells were evaluated by trypan blue exclusion test, flow cytometry, chromosomal aberrations, sister chromatid exchanges, the AMES test, cell cycle dynamics by mitotic inhibition and by flow cytometry. Data was analyzed by both one-way and two-way analysis of variance (ANOVA) when appropriate, t-student, Kruskall-Wallis and the Chi-squared test (X^2) based on data characteristics.

Results: A significant difference was obtained in the genotoxic activity of pristine CNTs when chromosomal aberrations (CA) and sister chromatid exchange (SCE) were evaluated in human lymphocytes. Ruptures in the cell's DNA were also evidenced by the comet assay on HUAEC and HepG2 cells when exposed to sub-lethal concentrations of both types of CNTs. Results on human lymphocytes, HepG2 and HUAEC for the biomarkers mentioned generated a significant difference when compared to the negative control. CNTs did not affect cell viability nor the cell cycle dynamics of HUAEC and HepG2 cells.

Conclusion: Nano-engineered materials such as CNT are attractive and ideal for different applications due to their many physical and chemical characteristics, however such characteristics also yield variable results when it comes to evaluating their effects on human cells. The variability is attributed to many factors including the target cell or tissue; as well as physico-chemical characteristics of the CNT. The results obtained are in agreement with several results previously reported; therefore, it is imperative to perform further in depth research prior to using them in the medical field.

1. Introduction

Cardiovascular diseases (CVDs) are a group of pathological conditions of the cardiovascular system, which compromise the heart, blood vessels and the pericardium. Most of these conditions involve the narrowing or blockage of the blood vessels that supply blood to the heart and brain. Among these conditions, coronary heart disease also known as ischemic heart disease is the most prevalent condition and it is associated with high mortality and morbidity. Cardiovascular diseases have become an issue of mayor interest due to their incidence in industrialized and developing countries. According to the World Health Organization, in the past decade cardiovascular diseases have remained the number one cause of death in the world. In 2012, an estimated 17.5 million people died from CVDs; of these deaths, approximately 7.4 million were due to coronary heart disease and 6.7 million were attributed to stroke [1]. One of the most often common techniques to treat cardiovascular disease is the use of stents, which are small metallic mesh tubes of different designs implanted in narrow or weak arteries [2].

Although stents have been proven to be effective in the treatment of cardiovascular diseases; there are still some risk factors associated with their use. Some of the risks include thrombus formation at the stent site and restenosis [3]. Since restenosis appears to be the mayor difficulty, it is imperative to solve the issue by researching new solutions. Implementation of nanotechnology in the medical field presents a solution to various health problems by improving the patient's health and quality of life. Some of the applications of nano-medicine include drug discovery and delivery agents, nano-robots and medical devices coated with nanostructured materials [4]. Although incorporation of carbon nanotubes in the fabrication of stents seems to be a plausible solution to efficiently treat cardiovascular diseases; there are areas of concern that need to be further evaluated. This project aimed at evaluating the effects of CNTs on human cells by assessing their potential risk hazard on the genetic material, since it is imperative to determine such effects before using CNTs on animals and humans. The results of this evaluation will contribute to the development and implementation of medical devices with the least biological risk, for instance a stent coated with nano-engineered material. This research is encompassed in the "Simulation by density functional theory (DFT),

molecular dynamics (MD) and computational fluid dynamics (CFD) techniques of the interaction of an endovascular nano-coating with lipoproteins present in the atherosclerotic plaque as a proposed method for therapeutic management."

2. Theoretical Framework

2.1 Endothelial Dysfunction

The vascular endothelium can be described as the inner lining of blood vessels and plays an important role in the circulatory system. Cells that make up the vascular endothelium are responsible for wall vascular function and respond to chemical signals produced by the body [5]. The endothelium acts as an active signal transducer and regulates tissue oxygen supply, vessel tone and diameter. Changes in the normal function of the endothelium lead to morphological alterations, lesion development, vascular disorders and clinical complications such as atherosclerosis [6], [7].

2.2 Percutaneous Coronary Intervention

Percutaneous coronary intervention also known as angioplasty is a non-surgical procedure in which a stent is used to unblock narrow arteries [2]. Treatment of obstructed arteries with coronary stent implants brings an immediate solution to open up the plaque-obstructed arteries [12]. Percutaneous interventions using a stent is a technique developed to decrease the complications associated with regular angioplasty and increase the blood flow capacity of obstructed arteries by supporting the vascular wall [3]. New approaches in drug eluting stent development have been proposed to achieve an improvement in stent technology and treat cardiovascular disease by minimizing or avoiding side effects which eventually compromise the patient's health [2], [13] [14]. A plausible technique for future stent development is the use of engineered nanostructured materials such as CNTs as a stent coating and drug delivery system.

2.3 Safety Evaluation of CNTs

Ever since the discovery of CNTs, their implementation in many areas has augmented due in part to their physico-chemical characteristics which make them a novel material

with a wide range of applications. Although the use of CNTs in the biomedical area is currently being explored and there are many possible ways in which this novel material could contribute to the development of new treatments, medical devices or diagnostic procedures; their long term effects and potential risks on human health are unknown or not sufficiently investigated [10],[11]. Therefore, prior to taking advantage of the many characteristics of CNTs and applying them in the biomedical area, *in vitro* and *in vivo* assays must be performed. Regulatory agencies such as the Organization for Economic Cooperation and Development (OECD) have stablished protocols to assess genotoxic and mutagenic effects. Those same protocols are employed to determine the damaging potential of CNTs on the genetic material, which would help to identify any hazards at the cell level. Other regulatory agencies such as the National Institute of Standards and Technology (NIST) have helped standardized methods and equipment to characterize nanomaterials, the Food and Drug Administration (FDA) provides regulatory guidance in the use of nanomaterials in food, cosmetics and animals; other government entities are promoting research to determine the exposure and its effects on the environment as well as in the occupational fields [11]. Since the field of nanotechnology is a promising solution to combat diseases such as cancer, the National Cancer Institute (NCI) has developed the Nanotechnology Characterization Laboratory to standardize protocols and methods involving the use of nanomaterials in order to define the effects on biological systems. Therefore, in order to overcome research obstacles, such as lack of available results for comparison purposes and lack of understanding the interaction of nanomaterials with biological systems [12]; the NCI developed an assay cascade which goes from performing physical characterization of the nanoparticle to *in vitro* and *in vivo* testing [13]. Apart from implementing all these necessary procedures, there are still gaps when it comes to the use of CNTs in the biomedical area, these gaps are related to appropriate dosage, route and time of exposure, variability in the results obtained, among deciding whether or not CNTs are safe.

2.4 Carbon Nanotubes

Carbon nanotubes are rolled up graphene sheets that form a cylindrical structure. CNTs are made up of arranged carbon atoms with diameters ranging from 1 to 100 nanometers;

and can be classified as single wall or multi wall tubular structures according to the arrangement of the carbon atoms [14]. Multi wall carbon nanotubes (MWCNTs) have several layers of graphene with a distance in between layers of 3.6 Å (Figure 1), and their electrical properties depend on the diameter and helical arrangement of the graphite rings [15]. Chirality or non-chirality of CNTs is determined by the positioning of the carbon hexagons in relation to the axis of the tube. A perpendicular position of the C-C bonds of the hexagon to the axis of the cylinder forms an armchair structure. Consequently, if the C-C bonds are parallel to the axis of the cylinder it forms a zigzag structure; lastly if the C-C bonds form an angle in relation to the axis of the tube it will be considered a helical arrangement better known as a chiral structure (Figure 2) [16]–[18]. CNTs are considered a very strong and stiff material, desired for its mechanical and electric properties. This material is very resistant and versatile therefore its applications are being explored in different industries [19]–[21].

CNTs can be produced through several methods such as laser ablation, electrolysis, solar energy, arc discharge and chemical vapor deposition, with the last two methods being the most commonly used. Growth of CNTs by chemical vapor deposition (CVD) is based on the thermal decomposition of hydrocarbons in the presence of a metal catalyst and a catalyst support [22], [23]. Mass production of CNTs can be achieved using this method of growth, since it is cost effective and the reaction time is significantly less when compared to the other methods.

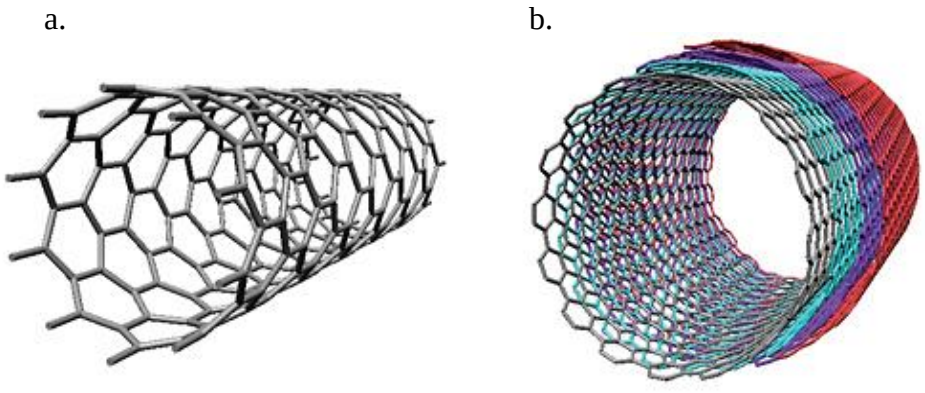

Figure 1. Schematic representation of: a) single-walled CNT and b) multi-walled CNT [18].

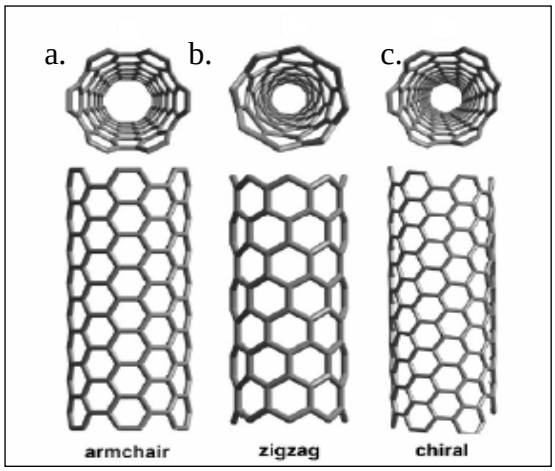

Figure 2. Structural types of single walled CNTs: a) armchair, b) zigzag and c) chiral [18].

2.5 Functionalization and Doping of Carbon Nanotubes

Carbon nanotubes are polar, hydrophobic, insoluble structures suitable for technological research due to their unusual mechanical properties; as a result, this innovative material must be modified in order to take full advantage of all its properties. Functionalization is a set of chemical reactions by which functional groups are covalently attached to the surface of the wall tube [24]. Functional groups make CNTs biocompatible and also add a new set of properties that are not present in unmodified (pristine) nanotubes. Another alternate method to change the structure of the wall tube is done by substitution reactions, also known as doping, where other atoms of different valence electrons (*i.g* nitrogen or boron) replace carbon atoms in the tube lattice. These substitutions usually change the balance state of the carbon nanotubes by either including electron-acceptor or electron-donor balance states [25]–[27]. Changes in the morphology and chemical reactivity have also been observed when nitrogen is used as a doping agent; nitrogen-doped nanotubes are extremely strong but have a lower oxidation resistance [28].

As previously mentioned, manipulation of the CNT lattice is possible by functionalization and doping; thus increasing their solubility and opening up a set of new properties that otherwise would remain unused. The use of nanotubes in medicine has become very popular due to the many possibilities for their use in diagnostics, drug delivery agents and implantable devices just to name a few [29]–[34]. CNTs are considered an optimal solution to improve the mechanism of action of stents,

incorporation of carbon nanotubes in stent developing provides the possibility to optimize stent performance by improving their functionality and possible diminishing or eliminating their side effects.

3. State of the Art

Sumio Iijima accidentally discovered carbon nanotubes in 1991 [14]; ever since this discovery new approaches for their synthesis and applications have been explored. The use of CNTs in the field of medicine and other industries has gained popularity due to their physicochemical properties. However, exposure to nano-engineered materials is of great concern due to the potential harm they pose on the environment and the human body. For instance, several researchers have emphasized the importance of studying the adverse effects of nano-materials on the environment and biological tissues. Extensive reviews have provided a better explanation of the many factors behind the nano-toxicology of engineered nanomaterials [35]–[38]. Physicochemical properties of nanoparticles such as size, chemical composition and surface structure exhibit adverse biological effects at the cellular level. Biological alterations such as genotoxicity and immunogenic changes are attributed to engineered nanoparticles, therefore approaches are currently being explored such as computer simulation to decipher and understand the interaction of CNTs with the cell [39].

Other researchers have identified vulnerable routes of exposure such as the skin, the respiratory and gastrointestinal track as semi-open surfaces with direct contact to the environment, facilitating the interaction of nanoparticles with biological tissues that could induce harmful effects [21]. Therefore studies conducted on rodents as well as on human cells have helped understand the mechanisms of toxicity of CNTs associated with their physicochemical characteristics and the health risks for humans [32]. Lam et al. [41] conducted an extensive review of several animal studies in which single-walled carbon nanotubes (SWCNTs) were tested to analyze lung toxicity. Lam et al. agreed that regardless of the process by which CNTs are synthesized, they all produce adverse effects in the lungs such as acute inflammation, oxidative stress, granulomas and fibrotic changes that are time and dose dependent. Researchers such as Lanone et al. [42]

reviewed the disturbing effects of CNT on the cell, and determined that severe inflammation and oxidative stress lead to redox imbalance which eventually translated into DNA damage [43]–[45]. It is thought that the toxic effects are due the surface chemistry and electrical properties of these nano-materials; which disturb the homeostasis of the cell by removing or shifting electrons. Autoimmune effects can be provoked when CNTs enter the cell and disturb the normal arrangement of the protein receptors. The original function of the protein is loss, and the secondary and tertiary structure of the protein is disrupted due to electrostatic and sterochemical binding of CNTs with proteins [36]. In addition to the physical disturbances, catalytic impurities also contribute to changes in cell homeostasis [46]–[48]. The review by Lam et al. [45] as well as reviews by other authors [34], [49] mention the possible side effects of manufactured SWCNTs and MWCNTs on cardiac toxicity and cardiopulmonary disease.

According to Ali-Boucetta et al. [49] the biodistribution and toxicokinetic profile of CNTs depend on factors such as route of exposure, molecular determinants and nature of functionalization. The authors observed that pristine SWCNT tend to accumulate in the liver, spleen and lungs while modified or functionalized CNTs are mostly secreted in the urine. On the other hand, exposure to carbon nanotubes through medical applications has shown an increase in cytotoxic and genotoxic effects on the human body. Li and coworkers [50] reviewed the biocompatibility and toxicity of different nanoparticles, giving special attention to CNTs. The use of CNTs in medicine as cancer treatments, biosensors [31], and drug and vaccine delivery systems has increased the interest to study their adverse effects on organs and tissues; as well the CNTs desirable outcomes and suitable benefits on local or systemic cellular response. Chemical properties and physical characteristics such as surface area, size, diameter, length and shape are factors that influence toxicity and biocompatibility.

Similarly, Jain et al [51] evaluated the characteristics of CNTs and their genotoxic effects at the cellular and systemic level. The information gathered by the authors explains how physical characteristics of CNTs interfere with the cell's genetic material often causing deleterious mutations. Formation of CNT aggregates in the cell results in inflammatory response, oxidative stress, tissue and organ destruction. Cytotoxic and genotoxic effects

of CNTs are also compared to those caused by asbestos, since size and shape of MWCNTs are similar to that of asbestos. Several authors have concluded that the toxic effects elicited by CNTs are of similar nature or even more dangerous than those caused by asbestos [51]–[53].

The surface area to size ratio of CNTs is one of the characteristics that make them extremely reactive and harmful to the cell; for instance, a nano-sized particle has the possibility to go undetected by the cell's innate defense mechanism [32], [54], [55]. If a phagocyte misses a nano-sized particle it could travel through the circulatory system and gain access to the nervous system. Potential systemic translocation of the nanoparticles from the cells to the circulatory system has been reported as a disadvantage of CNT accidental or predetermined CNT administration [50]. Systemic translocation is a topic of interest because CNTs can accumulate in the respiratory system when inhaled, or travel through the circulatory system and accumulate in other body regions. Nevertheless, Bussy et al. [56] investigated CNTs´ interaction with blood components and concluded that CNT surface characteristics (surface modifications, charge, structure and area) selectively interact with blood components; although functionalization or coating of CNTs improves biocompatibility it does not necessarily improve hemo-compatibility.

The interaction of CNTs with the circulatory system, specially blood and its components has also been studied by Bihari et al. [57], which evaluated the pro-thrombotic effects of SWCNTs and other two nanoparticles *in vitro* and *in vivo*. Platelet aggregation, p-selectin expression and platelet granulocytes complex formation were the characteristics observed. Their results showed that SWCNTs caused platelet aggregation and the production of thrombotic factors. A similar evaluation by Burke et al. [58] furthered research the thrombogenic effects of functionalized MWCNTs; the outcome of their analysis determined that CNT affected the clotting process in a dose dependent manner and cause thrombogenic effects in three different ways: factor independent platelet activation, activation of factor XII and binding of factor IXa.

The structure of CNTs can be changed, superficially or chemically modified through functionalization or doping. Modification of CNTs permits the addition of functional

groups and facilitates the interaction with biological tissue. Functionalized CNTs provide many advantages in the medical field, such as controlled dispersion of drugs and enhanced cell solubility thus reducing toxicity due to agglomeration. Although functionalization of CNTs increases their solubility, Firme and co-workers [59] report that the use of chemical substances employed in the modification of CNTs, as well as catalyst residue used in the synthesis of CNTs also produce toxic effects. The authors agree that toxic events caused by CNTs involve the activation of several biological pathways at the same time, leading to DNA destruction. In a different study conducted by Bottini et al. [60] two types of carbon nanotubes were tested on human T lymphocytes. Oxidized CNT and pristine SWCNTs were chosen for their different characteristics and applications. Cell viability and proliferation assays were conducted; it was found that oxidized CNT reduced cell growth more than pristine SWCNTs and both types of CNTs induced apoptosis in a dose and time dependent manner. As previously mentioned functionalized CNTs could be more toxic than hydrophobic unmodified CNTs due to their improved solubility and ability to disperse throughout the cell.

There is not a general guideline to pinpoint the source of toxicity of each type of CNTs on different biological tissues; therefore, cytotoxicity of CNTs depends on the mode of administration and the area of accumulation. The level of toxicity exerted could range from low to mild to severe, for this reason, CNTs designed for biomedical purposes must be meticulously evaluated at different levels (cell, organ and body systems).

4. Objectives

4.1 General Objective

The general objective of this research was to evaluate the risk hazards of CNTs in human cells by using a battery of bioassays to determine different genotoxic and mutagenic responses.

4.2 Specific Objectives

- Evaluate cell viability of endothelial and HepG2 cells treated with CNTs

- Assess genotoxic effects of CNTs in endothelial, HepG2 cells and human lymphocytes
- Evaluate the anti-proliferative effect of CNTs on endothelial and HepG2 cells
- Determine mutagenicity of CNTs in *Salmonella thyphimurium*.

5. Methodology

5.1 CNT Dispersion

Carbon nanotubes employed in this research were grown by chemical vapor deposition using Co_2O_4+Fe_2O_3 (40-10%) as a catalyzer at the Synthesis and Special Processes Laboratory at Universidad Pontificia Bolivariana. Both pristine and nitrogen doped (N-doped) CNTs tested had an average wall number between one and ten. The purification process of the CNTs samples was done by microfiltration with 10% hydrofluoric acid and hydrochloric acid [1M] resulting in a purity >95%. Characterization was achieved by micro-Raman and scanning electron microscopy (SEM) to verify the vibrational features and structure of the CNTs [61].

CNTs were dispersed in cell culture medium RPMI-1640 in order to test their effects on the cells. The dispersion process was achieved in the following manner: 1mg of the CNT sample (either pristine or N-doped) was autoclaved during 15 minutes at 121°C. Once sterilized, 10mL of RPMI-1640 were added to the sample, dispersion was achieved by using a 130-watt Cole-Parmer ultrasonic processor at amplitude of 50% during 2 minutes and 30 second pulsations. Sonication time, frequency and choice of equipment was determined based on the results reported by Hilding et al. [62]. Serial dilutions ranging from 0.01mg/mL to 0.1mg/mL with a dilution factor of 0.01 were prepared from the stock dispersion (1mg/10mL) of the pristine and the N-doped CNT samples. The stock dispersion and the serial dilutions were prepared fresh, once a month under strict sterile conditions. Presence of CNTs in the seriated dilutions was corroborated by scanning electron microscopy (SEM).

5.2. Cell Culture Maintenance

Experiments were performed using human endothelial cells derived from umbilical arteries (HUAEC), human liver hepatocellular carcinoma cells also known as HepG2, and human lymphocytes. For reasons that would later be specified, HUAEC cells were not used to determine chromosomal aberrations and sister chromatid exchange, human lymphocytes were used instead. All cell subcultures were performed under sterile conditions, using Endothelial Cell Growth Medium (EGM-2), Dulbecco´s Modified Eagle Medium (DMEM) and RPMI-1640 for the respective cell line. Subculture of the cells occurred once the cells reached a confluence of 80%. After assessing cell confluency, the depleted nutrient medium was discarded and the cell monolayer was washed three times with 4mL of 0.9% NaCl prepared with 500µl of 10.000 U/mL of penicillin-streptomycin. Trypsin EDTA was used to release the cell monolayer from the culture flask by adding 4 mL of 0.25% trypsin EDTA to the cell monolayer and returning the flask to the incubator for a period of 4 minutes. Detachment time was 2 minutes for HUAEC, human lymphocytes did not require to be detached. Elapsed the incubation time, the culture flask was quickly examined with an inverted microscope to verify cell detachment progress, when necessary, the culture flask was gently tapped against the palm of the hand to facilitate total cell detachment. Soon after cells were trypsinized, 4 mL of fresh medium prepared with 10% fetal bovine serum (FBS) was quickly added to the detached cells to stop the trypsin action. Cells were transferred to a 15mL sterile conical tube and centrifuged [63], [64].

HUAEC were centrifuged at 500 x *g* during 5 minutes and HepG2 cells were centrifuged at 200 x *g* during 7 minutes. Once centrifugation time was over, the supernatant was discharged and the cell pellet was re-suspended with 1mL of the respective cell culture medium supplemented with 2% FBS. Cell viability was evaluated on HUAEC and HepG2 cells with 2% trypan blue solution, viable cells appeared bright while non-viable cells appeared blue. Calculating the cell density per milliliter aid in determining the amount of cells to be seeded for each experiment, only cells with a viability above 90% were used to conduct the experiments. It is worth to mention that HepG2 cells employed in this research were sub-cultured up to 20 passages while HUAEC were propagated only up to 3 passages.

5.2.1 Endothelial Cell Isolation

HUAEC were isolated from human umbilical cord arteries by following an adaptation of the isolation method proposed by Jaffe et al. [65]. The umbilical cord was cut at the base of the placenta short after delivery, placed in a sterile 50mL conical plastic tube and stored at 4˚C when necessary until processing time. The cell isolation procedure was performed using sterile equipment and a class II type A biological safety cabinet. The outside surface of the umbilical cord was rinsed several times with a solution of PBS 1X in combination with of 10.000 U/mL of penicillin and 5mg/mL of gentamicin to remove excess blood. Once cleaned, the cord was inspected for ruptures and visible coagulated areas, hard coagulated portions of cord were cut and discarded. Each remaining fragment of cord was rinsed once with PBS 1X and the antibiotic mixture. The cord was clamped and the artery filled with type I collagenase at a concentration of 1mg/mL. The clamped cord was placed in a sterile petri dish, covered with aluminum foil and quickly transferred to a CO_2 incubator at 37˚C. After 15 minutes in the incubator, the cord was carefully removed from the petri dish and its contents emptied into a $75cm^2$ vented cap cell culture flask, to which 12mL of EGM-2 supplemented with 2% FBS were added. The isolated cells were placed in the CO_2 incubator and allowed to adhere to the cell culture flask during a period of 4-12 hours. After the adherence time elapsed, cells were washed with PBS 1X and the antibiotic mixture to remove any dead cells and remaining debris; once more an appropriate volume of EGM-2 supplemented with 2% FBS was added to the culture flask. The isolated HUAEC were kept in the CO_2 incubator until they reached their exponential state.

Characterization of endothelial cells was achieved by identification of CD31 or PECAM-1 (Platelet Endothelial Cell Expression Molecule-1) by flow cytometry and visual verification of their distinctive single cell layer “cobblestone” morphology when grown in culture [66]. CD31or PECAM-1 is a characteristic glycoprotein of endothelial cells involved in inter cellular junctions, cell adhesion and cell signaling. CD31 plays several important roles in the vascular biology, and has been widely used as an endothelial cell marker [67]–[70]. HUAEC were centrifuged twice after harvesting, and mixed with 100µl of PBS containing the IgG1 (isotype control) and the monoclonal antibody CD31-FITC (1:30, Invitrogen, MBC78.2). Cells were incubated during thirty minutes and

10.000 events per sample were acquired with the FacsCanto II (BD Biosciences, USA), data was analyzed using FlowJo X 10.0.7r2 version 1.6.

Vascular endothelial cells were chosen as one of the main cell types to access CNT effects on cytotoxicity and genotoxic due to their close interaction with blood, crucial role in vascular tone regulation, solute permeability from blood to tissues and non-thrombogenic surface [71], [72]. Also vascular endothelial cells are directly involved in complications such as arteriosclerosis, therefore they would be in direct contact with a nano-engineered medical device such a stent.

5.2.2 HepG2 Cell Line

HepG2 cells employed in this research were kindly provided by Programa de Estudio y Control de Enfermedades Tropicales (PECET) at Universidad de Antioquia. HepG2 is a human hepatoblastoma derived cell line, with a polygonal-like morphology; when in culture they exhibit monolayer growth in the form of "rosettes." This particular cell line was chosen because they preserve drug metabolizing enzymes important in the activation and detoxification of chemicals [73], [74]. HepG2 cells were propagated in DMEM supplemented with 10% FBS, incubated at 37˚C at an atmospheric humidity above 95% and 5% CO_2 [75].

5.2.3 Human Blood Samples

Five milliliters of peripheral blood were obtained from young healthy individuals after signing an informed consent. All blood samples were collected in the morning hours in heparin tubes and used within fifteen minutes of taking the blood sample. Lymphocytes were cultured with RPMI-1640 medium supplemented with 10% FBS, 500µl of 10.000 U/mL of penicillin, incubated at 37°C and stimulated with phytohemagglutinin (PHA) at a concentration of 0.2g/mL [76]. Human lymphocytes were chosen to perform the test because they are a type of white blood cell fundamental in the immune system which determine the specificity of the immune response to foreign substances or microorganisms [77], and as previously mentioned they would also be in direct contact with a nano-engineered stent.

5.3 Viability Assays

Cell viability was evaluated in HUAEC and HepG2 cells treated with CNTs by trypan blue exclusion test and flow cytometry.

5.3.1 Trypan Blue Exclusion Test

The trypan blue exclusion test is a commonly used method for cell viability analysis. This method is based on the principle that live cells have an intact cell membrane therefore the dye does not penetrate the cell; while dead cells take in the dye and appear blue [78]. This method is only good for differentiating dead from live cells; it is not suitable for identifying necrotic or apoptotic cells. The cell membrane integrity of HUAEC and HepG2 cells exposed to pristine and N-doped CNT dilutions was evaluated on three independent moments. HUAEC were seeded at a density of 8 x 10^4 cells per well, in a 6 well culture plate, 48 hrs. from the seeding time, cells were exposed to 40µl of each of the CNT dilutions (Section 5.1). After treating the cells with each dilution, cell cultures were incubated for an additional 24 hrs. Cells were harvested past 24 hours and immediately stained with a 0.2% trypan blue dye solution. The harvesting process was achieved by following steps described in section 5.2. DMEM was used a negative control for HepG2, and EGM-2 was used as a negative control for HUAEC and a 15% solution of H_2O_2 was used as the positive control for both types of cells. The same protocol was applied for HepG2 with the only difference that cells were seeded at a density of 5.0 x 10^4 cells per well; all evaluations were done for both types of cells on three independent moments with each type of CNT. Cell viability was calculated based on equation 1.

$$Cell\ viability = \frac{\#\ Viable\ cells}{Total\ Cells} \times 100 \qquad \text{Equation 1}$$

5.3.2 Cell Viability by Flow Cytometry

Cell viability by flow cytometry was determined by simultaneous staining with propidium iodide (PI) and 3,3'dihexyloxacarbocyanine iodide ($DIOC_6$). PI is a nucleic acid dye, which does not penetrate viable cells, while $DIOC_6$ is a cationic dye, which

strongly labels the mitochondria [79]. Both HUAEC and HepG2 cells were evaluated with the ten concentrations of the N-doped and pristine CNTs.

Cells were seeded at a density of 3.0 x 10^5 cells in 25cm^2 cell culture flasks; 48hrs from seeding time, cells were exposed to concentrations ranging from 0.01 to 0.1mg/mL of either type of CNT to be tested and incubated for an additional 24hrs at 37°C in a CO_2 incubator. DMEM was used a negative control for HepG2, and EGM-2 was used as a negative control for HUAEC and a 15% solution of H_2O_2 was used as the positive control for both types of cells. Cells were harvested past 72hrs from seeding time, by performing the previously described steps (Section 5.2). In order to stain the cells with PI and $DIOC_6$, the centrifuged cells were transferred to a 2mL vial, 200µl of PBS 1X were added to the vial in order to reach a final volume of 500µl (cells and PBS), to which 1µl of $DIOC_6$ [50mM] and 5µl of PI [50µM] were also added. After adding the dyes, cell samples were incubated at room temperature, in the dark during 20 minutes. Data was acquired for 100.000 events per sample using FacsCanto II (BD Biosciences, USA) flow cytometer; viability was evaluated for both types of cells on three independent moments with each type of CNT. Data was analyzed using FlowJo X 10.0.7r2 version 1.6.

5.4 Genotoxicity Assays

Genotoxicity is the ability of a chemical or physical agent to interact with the DNA, RNA or other genetic material in the cell, often resulting in an alteration, insertion, rearrangement, or deletion of the structure of a gene or entire chromosomes. All genotoxicity assays were performed by testing three sub-lethal concentrations (0.08, 0.09, and 0.1mg/mL) of both CNT samples. Only concentrations that yield viability results above 80% were chosen to perform the genotoxicity assays with the purpose of avoiding false positive results [80]. The sub-lethal concentrations were chosen based on viability tests results obtained by trypan blue and flow cytometry evaluations; as well as concentrations reported by other authors [52], [59], [81]. Although, all the concentrations evaluated by trypan blue exclusion test and by flow cytometry (0.01mg/mL - 0.1mg/mL) yielded results above 80%; only the three highest concentrations were chosen. Chromosomal Aberrations (AC), sister chromatid exchange (SCE) and single cell gel

electrophoresis (SCGE) were the biomarkers chosen to determine DNA damage on HUAEC, HepG2 cells and human lymphocytes.

5.4.1 Chromosomal Aberrations

Structural changes to one or more chromosomes by a genotoxin can be determined by the chromosomal aberration assay [82]. Structural damages of chromosomes were classified as chromatid break (ctb), chromosome break (csb), dicentric chromosome (dic), ring (r) and multi-radial figures (mr).

HepG2 and human lymphocytes were used to perform the chromosomal aberration (CA) analysis. HUAEC were not employed in this assays because it was impossible to obtained chromosomal extends due to cell death upon exposure to the mitotic spindle inhibitor. Cells were maintained under previously discussed culture conditions (Sections 5.2). HepG2 cells were seeded at a density of 5.0 $x10^4$ cells per well in a 6 well culture plate, exposed to sub-lethal concentrations [0.08, 0.09 and 0.1mg/mL] of N-doped and pristine CNTs. Treatment of human lymphocytes was performed in the same manner as to HepG2 cells, but exposure to CNTs was 36hrs from seeding time instead of 48hrs. Two hours before harvesting, cell cultures were treated with Colcemid [10ug/mL], a mitotic spindle inhibitor, to induce mitotic arrest. Separation of chromosomes was achieved by performing the wet plate dripping technique. Cells were treated with a hypotonic solution of 0.7% sodium citrate and centrifuged at 200 x *g* during 7 minutes. Subsequently, cells were fixed twice with methanol/acetic acid in a 3:1 ratio. Cell dripping was performed using glass slides, which were previously cleaned and cooled. Staining of cells was done with a 5% Giemsa solution during 7 minutes [63]. One hundredth mitotic cells were analyzed per treatment with an Olympus optical microscope using an objective of 100X, with the purpose of classifying and quantifying CA. RPMI-1640 was used as a negative control for human lymphocytes, DMEM was used as negative control for the established cell line HepG2 and mitomycin-c [10μM] was used as a positive control [63],[60], [83]. Basal damage in a healthy individual has been previously reported by other authors to be between 2 and 5% [84], [85], [86].

5.4.2 Sister Chromatid Exchange

Another cytogenetic technique used to evaluate chromosomal damage is the sister chromatid exchange (SCE). This technique permits to identify the exchange of DNA material between sister chromatids in cells that completed two cell cycles (M2). This assay is performed by incorporating 5-bromo-2-deoxiuridine (BrdU) an analogue of thymine during the replication of the genetic material. BrdU permits the direct visualization of genetic exchanges between the chromatids breaks, which are evidence of DNA ruptures that could have been correctly or incorrectly repaired. These changes are visualized as unexpected interruptions in the DNA sequence when a differential coloration of the chromosomes is done [87]. HUAEC and HepG2 cells were originally intended to be used in this assay, but due to unexpected difficulties such as cell death after exposure to the spindle inhibitor in HUAEC and difficulties with the differential coloration in HepG2 it was impossible to evaluate SCE on these two types of cells therefore, human lymphocytes were used instead.

Human lymphocytes were incubated 48 hours, marked with BrdU [1mg/mL] and stimulated with PHA as previously mentioned (Section 5.2.3). Exposure to CNT dilutions of [0.08, 0.09 and 0.1 mg/mL] were done during 24 hours and two hours before harvesting the cells, the spindle inhibitor (Colcemid [10ug/mL]) was used to induce mitotic arrest. Cells were treated with a hypotonic solution of 0.7% potassium chloride and centrifuged at 200 x *g* during 7 minutes; posterior to this, cells were fixed twice with methanol/acetic acid in a 3:1 ratio. Separation of the chromosomes was achieved by performing the wet slide dripping technique. Cell dripping was performed using glass slides, which were previously cleaned and cooled. Differential coloration was carried out by soaking the dripped slides in Hoechst 33258 during 10 minutes, then rinsed with distilled water and illuminated with a 120w lamp during 20 minutes at 65°C. The slides were kept moist with a 2XSSC buffer solution while exposed to direct light. Staining of cells was done with a 5% Giemsa solution for approximately 3 minutes. The amount of sister chromatid exchanges was scored only for cells that were on the second cycle of metaphase, SCE were identified in 50 cells and the results were shown as the frequency of sister chromatid interchange per metaphase. As a negative control, RPMI-1640 was used and mitomycin-c [10μM] was used as a positive control, the experiment was

performed three times on different occasions. The frequency of SCE in a healthy individual has been previously established to be between 5-7 SCE in 30 cells [85].

5.4.3 Single Cell Gel Electrophoresis

Single cell gel electrophoresis (SCGE) also known the Comet assay as is a powerful technique that permits to evaluate single and double strand DNA breaks on individual cells. This methodology permitted to evaluate DNA damage in HUAEC and HepG2 after treating them separately with three sub-lethal concentrations [0.08, 0.09 and 0.1mg/mL] of both types of CNTs. The comet assay consists of separating and characterizing DNA that migrates out of the cell [88]. This process involves the immobilization of cells by embedding them in agarose gel (GelBond® film) with low melting point agarose (LMPA), then the agarose gel is submerged in a lysing buffer to achieve cell membrane rupture, the DNA migrates to the anode when exposed to current during electrophoresis leaving a trail of rupture DNA behind "comet tail." Samples were stained with GelRed™ for better visualization of the comet tail. The results of the test were read by measuring the length of the tail. The comet assay has proven effective for the identification of strand breaks, alkaline-labile site damage and mismatch repair site [89], [90].

HUAEC and HepG2 cells were seeded in 6 well plates at a density of $1.0x10^5$ and $1.0x10^4$ respectively, under previously described conditions. Forty-eight hours from the seeding time, treatment of the cells with the three sub-lethal concentrations [0.08, 0.09 and 0.1mg/mL] corresponding to either type of CNTs were done. Cells were exposed to CNTs during 24 hours, harvesting occurred as described on sections 5.2.1 and 5.2.2. Post treatment cell viability was evaluated with 0.2% trypan blue dye solution (Section 5.3.1). Cell suspensions were mixed with LMPA, embedded in the agarose gel film and placed in the lysing solution for 16 to 24 hours. The lysis solution contained 100mM EDTA, 0.05% SDS, 2.5M NaCl, 1% Triton X-100, 10mM Trizma-base and 10% DMSO added prior to its use, the pH was adjusted to 10 with NaOH. The high concentration of salt in the lysis solution helps to break down the cell membrane, remove histones and other soluble proteins [91]. After the cells were lysed, the agarose film was placed in a horizontal electrophoresis chamber and covered with cold electrophoresis buffer pH>13 (often containing 300mM NaOH, 1mM EDTA) during 40 minutes. After 40 minutes, the

agarose film with the embedded cells was electrophoresed for 30 minutes at 25mV and 300 mA. The agarose film was removed from the electrophoresis chamber, rinsed with neutralizing buffer and set aside in a draining plate, dehydration of the embedded LMPA cells was done with methanol prior to staining. Cells were stained with GelRed™ [0.01X] for approximately 10 seconds and rinsed with distilled water to remove excess dye.

The agarose film was cut and mounted on glass slides; samples were analyzed with an Olympus fluorescent microscope and a 40X objective. DMEM was used as a negative control and H_2O_2 [50μM] was used as a positive control. The comet assay was done on two independent moments with two replicates each time it was performed; two hundred cells were analyzed per treatment. DNA damage was measured based on the length (μm) of the DNA fragments that migrated out of the cell (comet tail). Damage was quantified based on four categories [92] which were calculated as follow: no damage corresponds to the average of negative control + 1 SD of negative control, low damage corresponds to superior limit of previous level + 1 SD of negative control, moderate damage corresponds to the superior limit of low damage + 1 SD of negative control, and total damage was determined to be above the superior limit of moderate damage level (Table 1).

Table 1. Damage levels (average comet tail length) in HUAEC and HepG2 cells treated with pristine and N-doped CNTs during 24hrs.

	Compound	No Damage	Low Damage	Moderate Damage	Total Damage
HepG2	Pristine CNT	25.7-31.6	31.7-37.6	37.7-43.6	> 43.6
	N-doped CNTs	28-34.1	34.2-40.3	40.4-46.5	> 46.5
HUAEC	Pristine-CNT	22.6-28.4	28.5-34.3	34.4-40.2	> 40.2
	N-doped CNTs	23.8-29.3	29.4-34.9	35-40.5	> 40.5

Data reported in this table corresponds to average damage scored in 200 cells per treatment, cell culture media was used as negative control.

5.5 Life Cycle Dynamics

Performing an extensive analysis of the cell's life cycle permits to identify cell cycle variations caused by the addition of specific treatments or external factors. Time related events and modifications induced by an external factor can be associated to a specific cell

cycle phase [93], [94]. Due to the sensibility of HUAEC to mitotic inhibitors such as colcemid and colchicine, the cell cycle analysis of HUAEC was performed by flow cytometry; while the duration of the cell cycle of HepG2 cells was assessed by accumulation function.

5.5.1 Cell Cycle Dynamics by Accumulation Function

Cell cultures of HepG2 were treated during 24 hours with the highest concentration (0.1mg/mL) of either nitrogen-doped or pristine CNTs. The cell cycle was monitored every hour, by exposing the cells to a mitotic spindle inhibitor (Colcemid, [10ug/mL]); and the mitotic index was determined every hour during 30 hours. The life cycle dynamics of HepG2 was performed twice at two independent moments; the duration of the cell cycle for HepG2 was determined by plotting the results obtained after solving Puck & Steffen's equation [93].

$$Log(N_M + 1) = \frac{(0.301)(T_M + t)}{T} \qquad \text{Equation 2}$$

Where N_M is the mitotic index, T_M is the time of mitosis, t is the time cells were exposed to the mitotic spindle inhibitor and T is the generation time.

5.5.2 Cell Cycle Analysis by Flow Cytometry

HUAEC cells were seeded at a density of 1.0 x 10^6 cells per flask, and exposed to all ten concentrations (0.01 to 0.1mg/mL) of either nitrogen-doped or pristine CNT during 24h. Cells were harvested by following the previously described methodology (Section 5.2) and fixed in 90% cold ethanol added drop wise to the cell pellet while vortexing. Cells were fixed for 2hrs at 4°C, then washed twice with 1X PBS, treated with PI [0.1mg/mL] and incubated for 30 minutes at room temperature protected from direct light [95], [96]. Cell cycle analysis of HUAEC by flow cytometry shows quantitation of DNA content corresponding to the amount of DNA in cells during each phase of the cell cycle (G1/G0, S, G2/M). The technique was performed twice on two different occasions; data was analyzed with the flow cytometry analysis software ©FlowJo version x.0.7.

5.6 Mutagenicity Assay

The bacterial reverse mutation test also known as the Ames test was used to identify DNA mutations induced by CNTs. This assay requires the use of mutant (histidine dependent) *Salmonella thyphimurium* strains which help detect substances that alter the genetic material. A substance or compound is identified as a mutagen because it reacts with the histidine dependent strains, allowing them to grow in a histidine free environment. In order for a compound or substance to be considered mutagenic it must meet the following two criteria: it must yield a direct and proportional relationship between the number of revertant colonies and the dose administered, and it must produce the same results on at least two independent occasions and at the very least in one or two of the bacteria strains [97].

Strain checks of *S. thyphimurium* TA98 were done previous to performing the test, in order to confirm the genetic integrity and spontaneous mutation rate of the strain. The TA98 strain has the *his*D gene mutated, which encodes for histidinol dehydrogenase enzyme; the mutated *his*D3052 allele has a 1 frameshift mutation closely positioned to the *his*D3052 operon, affecting the reading of the G-C sequence which repeats four times [98],[99]. Therefore, TA98 strain was used to identify the ability of the CNTs to cause a reverse frameshift mutation on the affected allele.

S. thyphimurium strain TA98 was grown overnight in liquid nutrient broth during 16hrs in a constant shaker incubator at 37˚C and approximately 80 rpm. Optical density (OD) measurement was done at 650nm to confirm bacterial growth. Only bacteria with optimal readings between 0.5 and 0.6 were considered for the test, indicating the presence of approximately $1.0x10^7$ - $1.0x10^8$ bacteria in the liquid broth. Once bacteria reached the optimal OD readings, a mixture of sub-lethal concentrations [0.08, 0.09 and 0.1mg/mL] of either type of CNTs and bacteria were incubated at 37˚C during 90 minutes. Following incubation, top agar was added to the treated bacteria; the preparation was poured into minimal bottom agar plates and incubated during 72 hours at 37˚C.

Sub-lethal concentrations were obtained after performing a viability test on strain TA98 with concentrations varying from 10mg/mL to 0.01mg/mL. These treatments were done in the presence and absence of metabolic activation factor S9 [100]. The procedure was performed twice on three different occasions. The mutagenicity index (MI) was used to quantify the mutagenicity of CNTs on the bacterial DNA. The MI was calculated using equation 3.

$$MI = \frac{Average\ of\ revertant\ colonies\ per\ treatment}{Average\ of\ revertant\ colonies\ in\ the\ negative\ control} \quad \text{Equation 3}$$

The compound tested was categorized based on the mutagenicity index as a strong mutagen (MI >2), weak mutagen (MI range between 1 and 2) or non-mutagen (MI ≤1) [97], [101].

6. Statistical Analysis

Data analysis was done with IBM SPSS Statistics version 23 IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. and GraphPad Prism version 6.3 for Macintosh, GraphPad Software, La Jolla California USA. All data was subjected to either Kolmogorov-Smirnov, D'Agostino & Pearson omnibus normality test or Shapiro-Wilk normality tests prior to analysis.

Data collected for the first specific objective of this research, which was to evaluate cell viability by trypan blue exclusion test and by flow cytometry was analyzed by both one-way and two-way analysis of variance (ANOVA) when appropriate, t-student test was used for data that were normally distributed, and the Kruskall-Wallis test for data that did not pass the normality test. Both viability evaluation methods were assigned a response variable which corresponds to live cells and two independent variables which correspond to CNT type and concentration.

Results obtained from the genotoxic tests performed to assess DNA damage (CA and SCE) correspond to the second specific objective and were evaluated by the non-parametric Chi-squared test (X^2). Since not a sufficient amount of damage was found, the

response variable (damage) was analyzed as a qualitative variable instead of quantitative variable; hence damage percentage was assessed. The results obtained with the comet assay, which also responds to the second objective, were analyzed with the Kruskall-Wallis test.

The third specific objective was performed to determine the mutagenicity of CNTs on *S. thyphimurium*, the response variable was determined to be the number of revertant colonies and the independent variables were: the presence or absence of S9, the type of CNT and the concentration tested. One-way ANOVA in conjunction with the Tukey test were used to compare the groups of data. It is worth to mention that every time ANOVA was performed, Levene's test was used to assess the variance homogeneity prior to performing the analysis.

Evaluation of the cell cycle dynamics accounts for the fourth specific objective of this research, cell cycle data of HUAEC obtained by flow cytometry was analyzed with the Kruskall-Wallis test; the response variable was identified as the amount of DNA per cell in each phase of the cell cycle and the independent variables were the type of CNT and concentration. The cell cycle dynamics information collected from HepG2 cells was not analyzed with any statistical test, instead the duration of the life cycle was calculated by obtaining an accumulation function and subsequently the mitotic index (response variable) of cells subjected to the highest concentration of both types of CNTs (independent variable). The results were subjected to Puck & Steffen's equation (equation 2), a logarithmic calculation was obtained and a plot of the accumulation function was done, from this plot the effect of CNTs on the duration (hours) of the cell cycle of HepG2 was determined.

7. Results

7.1 CNT Dispersion in Cell Culture Media

Evidence of the synthesis, purification and dispersion of the CNTs used in the biocompatibility assays are shown in figures 3-5. Figure 3a shows a SEM micrograph of intact N-doped CNT before being sonicated, it is important to note that CNTs appear clumped and tangled (see arrows). Figures 3b and 3c show the dispersed N-doped CNTs

[0.1mg/mL] samples after being autoclaved and sonicated during 2 minutes in cell culture medium. Raman spectra of N-doped CNT catalyzed from cobalt is shown in figure 4. The relationship of intensity between band I_D and I_G with a value of 1.15 a.u corroborates the presence of nitrogen in the lattice of the cobalt grown CNTs. The ideal value of the I_D/I_G ratio for pristine CNTs is 0.45 a.u, which means that the CNT lattice is solely composed of carbon atoms, therefore an increment in the I_D/I_G confirms the substitution of carbon atoms in the nanotube structure by an atom different than carbon; in this case some carbon atoms were substituted by nitrogen atoms [102].

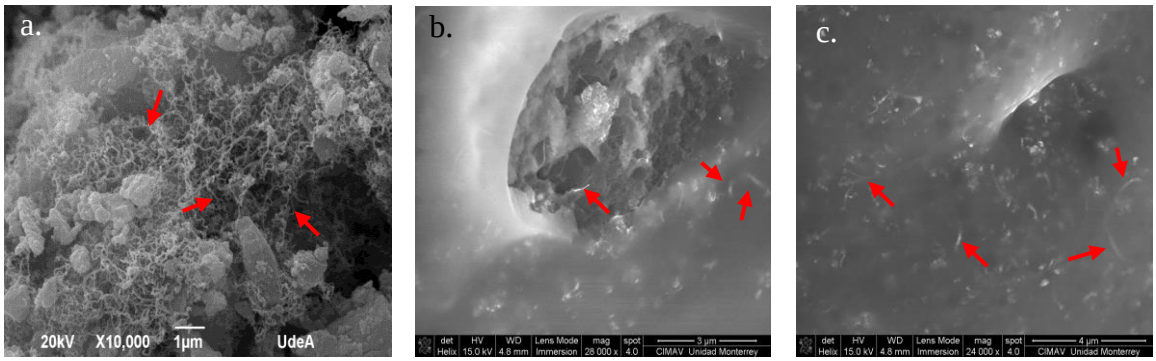

Figure 3. SEM micrographs of a) N-doped CNT grown from Cobalt before dispersion, b) and c) N-doped CNT [0.1mg/mL] dispersed in RPMI-1640, with an average size of less than1µm, image magnified at 28000X and 24000X respectively. Source: Dr. Lina Marcela Hoyos Palacio.

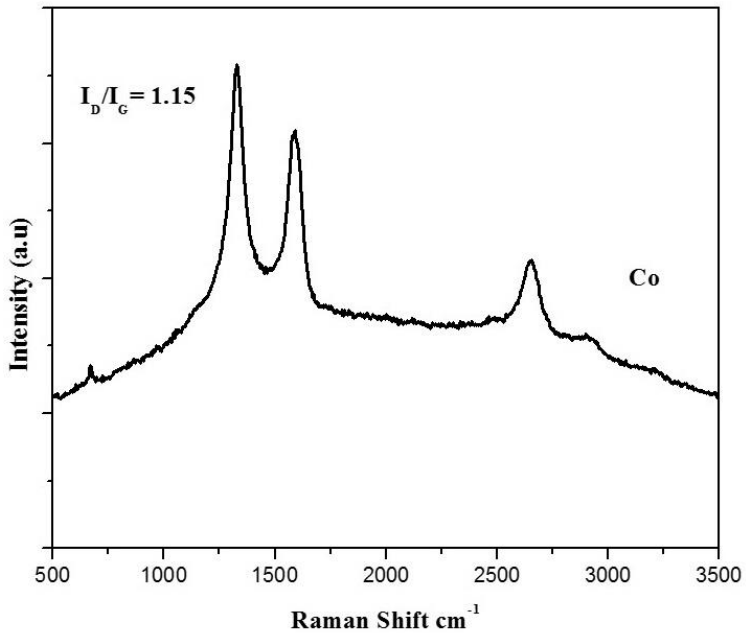

Figure 4. Raman spectra of N-doped CNTs grown from Cobalt. I_D shows the diameter and wall thickness of the CNTs, I_G shows the presence of carbon atoms. Source: Dr. Lina Marcela Hoyos Palacio.

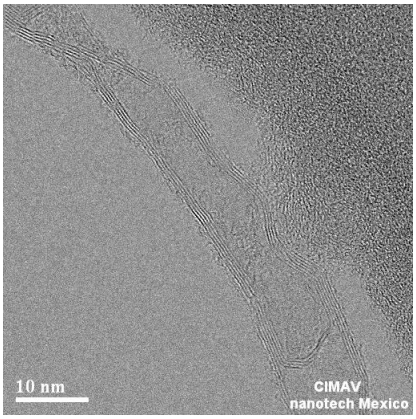

Figure 5. TEM micrograph of multi walled CNTs grown from Co. Average wall number ranging between 1 and 10 walls. Source: Dr. Lina Marcela Hoyos Palacio.

7.2 Cell Culture Maintenance

7.2.1 Endothelial Cell Identification

Endothelial cells isolated from human umbilical arteries were identified by flow cytometry immunophenotyping by detection of surface cell marker CD31/PECAM-1. Histograms shown are representative of endothelial cell identification performed on cells isolated from different umbilical cords, CD31 detection was always performed on the first population doubling. Figure 6a shows the flow cytometry histogram for the primary non-specific IgG1 isotype (negative control), while figure 6b show that 90.9% of cells tested positive for CD31.

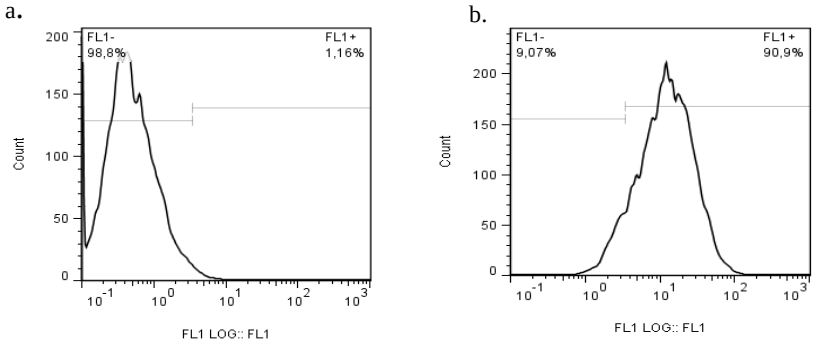

Figure 6. Flow Cytometry Histograms of HUAEC a) Isotype control b) percentage of HUAEC cells expressing CD31 cell membrane surface marker.

7.2.2 HUAEC and HepG2 Cell Morphology

Morphological characteristics of HUAEC (figure 7a) and HepG2 (figure 7b) are clearly distinguished in the photographed cell cultures. Both cell types grow in monolayer but have a distinctive growth pattern.

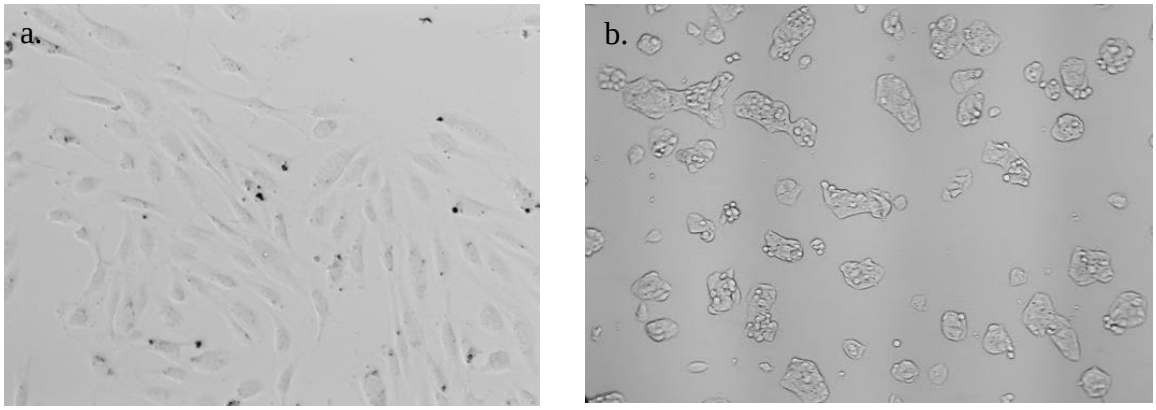

Figure 7 a) Endothelial cell "cobblestone" morphology when grown in culture and treated with [0.05 mg/mL] N-doped CNTs; 7b) untreated cell culture of HepG2 with its polygonal like morphology and characteristic "rosette" monolayer arrangement. Source: Grupo Biología de Sistemas at UPB.

7.3 Viability Assays

7.3.1 Trypan Blue Exclusion Test on HUAEC and HepG2

Cell viability was evaluated by trypan blue exclusion test on HUAEC and HepG2 cells after being treated with pristine and N-doped CNTs at different concentrations during 24hrs. Percent viability of both types of cells was above 80% for all the concentrations tested, it is worth to note that there is no significant difference in the cell viability of both types of cells when compared to the percent viability of the negative control (93%). Figure 8 shows the percent viability of each type of cell exposed to the lowest [0.01mg/mL] and the highest [0.1mg/mL] concentrations of N-doped and pristine CNTs. There was no significant change in the percent viability of the cells exposed to the ten concentrations of CNTs. The p-value obtained when a comparison was made between cell type, concentration and CNT type was 0.716 which indicates no significant difference between any of the factors compared. There is also no interaction between the CNTs and the type of cell used, p-values 0.177 and 0.196 respectively; which means that cell viability was not affected regardless of the type of cell or the type of CNT used. A comparison of cell viability based on the amount of CNTs used did show a significant

difference; p-values of 0.004 and 0.005 for pristine and N-doped CNTs respectively; although the different concentrations do make a difference in the cell viability, the percentage of viable cells remained above 80% regardless of the concentration evaluated.

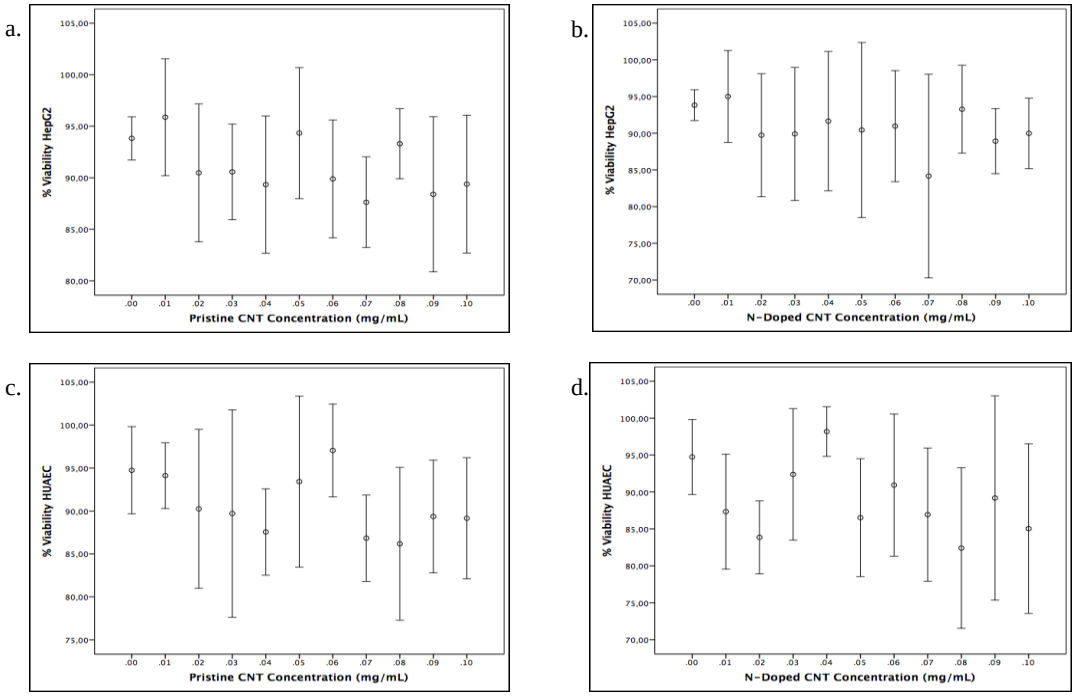

Figure 8. Cell viability by trypan blue exclusion test. HepG2 exposed to ten concentrations of a) pristine CNTs and b) N-doped CNTs. HUAEC exposed to ten concentrations of c) pristine CNTs and d) N-doped CNTs. Cells were exposed to different concentrations of CNTs during 24h. RPMI-1640 was used as a negative control and a solution of H_2O_2 (15%) was used as a positive control for all experiments, p-value = 0.716.

7.3.2 Cell Viability by Flow Cytometry on HUAEC and HepG2

Cell viability results by simultaneous staining with PI and $DIOC_6$ evaluated by flow cytometry are reported in figure 9, which shows cell viability and mitochondrial membrane potential of both types of cells 24h after exposure to concentrations ranging from 0.01 to 0.1mg/mL of pristine and N-doped CNTs. Figures 9a and 9b correspond to HUAEC while figures 9c and 9d represent HepG2 cells. Figure 9a shows the percentage of HUAEC positive for $DIOC_6$ and PI exposed to N-doped CNTs. It is worth to note that neither $DIOC_6$+ nor PI+ cells were significantly different when compared to the negative control (p=0.7734 and p=0.7658 respectively). Figure 9b corresponds to HUAEC exposed to pristine CNTs, the cell percentage positive for $DIOC_6$ had a non-significant p-value of 0.0905, the same trend was observed for cells that tested positive for PI with a

non-significant p-value of 0.1615 when compared to the negative control. HepG2 cells were also exposed to N-doped CNTs (figure 9c), the percentage of cells which tested positive for $DIOC_6$ (p-value =0.3463) do not show a significant difference when compared to the negative control; while the p-value of PI+ cells was 0.0423 which is significantly different when compared to the negative control. Lastly HepG2 cells exposed to pristine CNTs (figure 9d) had a non-significant p-value of 0.6728 for $DIOC_6$+ cells, as well as PI+ cells which had a p-value of 0.6853, again not significant when compared to the negative control.

A comparison between the percentage of live cells ($DIOC_6$+) obtained when both HUAEC and HepG2 cells were exposed to pristine and N-doped CNTs is seen in figure 10. Figure 10a corresponds to HUAEC while figure 10b corresponds to HepG2; there is a significant difference (p-value=0.0020) in the percentage of $DIOC_6$+ cells exposed to 0.03mg/mL of N-doped CNTs when compared to the negative control (figure 10b). This significant difference is evident in figure 9c which corresponds to Hepg2 cells exposed to 0.03mg/mL of N-doped CNTs, p=0.0423 corresponding to PI.

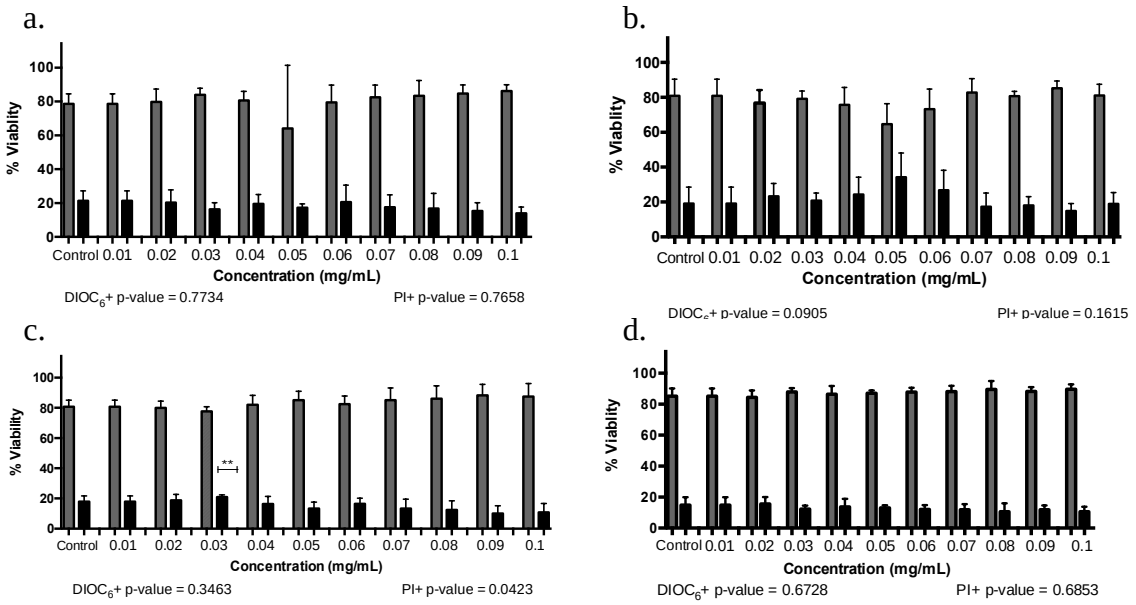

Figure 9. Cell viability by flow cytometry, cells were treated with different CNT concentrations during 24h, RPMI-1640 was used as a negative control and a solution of H_2O_2 (15%) was used as the positive control. Grey bars correspond to %$DIOC_6$+ and black bars correspond to %PI+ cells. a) HUAEC exposed to N-doped CNTs. b) HUAEC exposed to pristine CNTs, c) HepG2, exposed to N-doped CNTs d) HepG2 exposed to pristine CNTs.

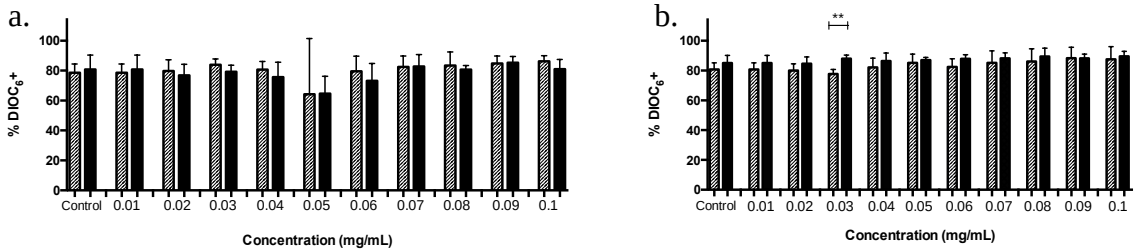

Figure 10. Changes in mitochondrial membrane potential detected with $DIOC_6$ for both cell types exposed to different concentrations of N-doped CNT (pattern bars) and pristine CNT (solid bars). RPMI-1640 was used as a negative control. Cells were exposed to different CNT concentrations during 24h. a) HUAEC p-value =0.9999, b) HepG2 p-value = 0.0020.

7.4 Genotoxicity Assays

7.4.1 Chromosomal Aberrations in Human Lymphocytes

Chromosomal aberrations found in human lymphocytes treated with sub-lethal concentrations of pristine and N-doped CNTs [0.08, 0.09 and 0.1mg/mL] during 24hrs are reported in table 2. A significant difference was observed in the two types of damage: chromatid break and chromosome break in human lymphocytes exposed to pristine CNTs [0.1mg/mL] compared to the negative control p-values of 0.011 and 0.001, respectively. Thirteen out of two hundred scored cells exposed to 0.1mg/mL of pristine CNTs had single strand breaks, which means that 6.50% of the chromatids were broken, while 5.50% of chromosomes in 200 scored cells exposed to the same concentration of pristine CNTs exhibit double strand breaks. The percentage of chromatid and chromosomal breaks found in cells exposed to lower concentrations (0.08 and 0.09mg/mL) was also significantly different when compared to the control. Only 4.12% of single strand breaks and 4.71% of double strand breaks were found in cells exposed to 0.08mg/mL of pristine CNTs. The percentage of damage for chromatid breaks was only 1% in cells exposed to 0.09mg/mL pristine CNTs, no chromosomal breaks were found. There was no presence of other type of damage such as multi-radial figures (p=0.272) and ring chromosomes (p=0.779) in human lymphocytes treated with pristine CNTs. On the other hand, N-doped CNTs did not cause any type of significate damage on human lymphocytes (p>0.064).

The chromosomal aberrations identified in HepG2 cells treated with sub-lethal concentrations of pristine and N-doped CNTs [0.08, 0.09 and 0.1mg/mL] during 24hrs

are (Table 3). No significant difference was observed for any type of damage when comparing the results for the treated cells with the negative control ($p>0.6$).

Table 2. Chromosomal aberrations identified in human lymphocytes exposed to N-doped CNTs [0.08, 0.09 and 0.1mg/mL] and pristine CNTs [0.08, 0.09 and 0.1mg/mL] during 24h. RPMI-1640 was used as the negative control and mitomycin-c [10μM] was used as the positive control.

Compound	Concentration (mg/mL)	ctb				csb				dic/r				mr			
		No damage		Damage		No damage		Damage		No damage		Damage		No damage		Damage	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Control	0.00	200	100.0	0	0.00	199	99.5	1	0.50	197	98.5	3	1.50	199	99.5	1	0.50
Pristine CNT	0.08	163	95.9	7	4.12	162	95.3	8	4.71	169	99.4	1	0.59	170	100.0	0	0.00
	0.09	198	99.0	2	1.00	200	100.0	0	0.00	194	97.0	6	3.00	199	99.5	1	0.50
	0.1	187	93.5	13	6.50	189	94.5	11	5.50	196	98.0	4	2.00	198	99.0	2	1.00
Chi-squared value		0.011				0.001				0.272				0.779			
N-Doped CNT	0.08	200	100.0	0	0.00	200	100.0	0	0.00	196	98.0	4	2.00	199	99.5	1	0.50
	0.09	185	98.9	2	1.07	185	98.9	2	1.07	178	95.2	9	4.81	185	98.9	2	1.07
	0.1	198	99.0	2	1.00	199	99.5	1	0.50	198	99.0	2	1.00	198	99.0	2	1.00
Chi-squared value		0.473				0.318				0.064				0.870			

ctb: chromatid break, csb: chromosome break, dic: dicentric chromosome, r: ring and mr: multi radial figure.

Table 3. Chromosomal aberrations identified in HepG2 cells exposed during 24hrs to N-doped and pristine CNTs [0.08, 0.09 and 0.1mg/mL], RPMI-1640 was used as the negative control and mitomycin-c [10μM] was used as the positive control.

Concentration (mg/mL)		ctb				csb				dic/r				mr			
		No damage		Damage		No damage		Damage		No damage		No damage		Damage			
		n	%	n	%	n	%	n	%	n	%	n	%	N	%		
Control	0.00	39	97.5	1	2.50	39	97.5	1	2.50	40	100.0	40	100.0	0	0.00		
Pristine CNT	0.08	153	96.2	6	3.77	157	98.7	2	1.26	159	100.0	159	100.0	0	0.00		
	0.09	100	97.1	3	2.91	101	98.1	2	1.94	103	100.0	103	100.0	0	0.00		
	0.1	161	95.8	7	4.17	166	98.8	2	1.19	168	100.0	167	99.4	1	0.60		
Chi-Squared value		0.856				0.913				indeterminate		1.0					
N-Doped CNT	0.08	191	95.5	9	4.50	197	98.5	3	1.50	200	100.0	200	100.0	0	0.00		
	0.09	187	95.4	9	4.59	195	99.5	1	0.51	196	100.0	196	100.0	0	0.00		
	0.1	189	94.5	11	5.50	198	99.0	2	1.00	200	100.0	200	100.0	0	0.00		
Chi-Squared value		0.608				0.950				indeterminate		indeterminate					

ctb: chromatid break, csb: chromosome break, dic: dicentric chromosome, r: ring and mr: multi radial figure.

7.4.2 Sister Chromatid Exchange in Human Lymphocytes

N-doped CNTs caused a significant amount of sister chromatid exchange in cells exposed to all three concentrations, while cells exposed to pristine CNT were significantly affected by only one of the concentrations p-value = 0.0001 when compared to the negative control. (Table 4 and Figure 11).

Table 4. Sister chromatid exchange in human lymphocytes treated with pristine and N-doped CNTs during 24hrs, RPMI-1640 was used as a negative control and mitomycin-c [10μM] was used a positive control.

Compound	Concentration (mg/mL)	Min	Max	Mean	SE	Median	P25	P75
Pristine CNTs	Control	0.0	1.0	0.2	0.4	0.0	0.0	0.0
	0.08	0.0	7.0	0.5	1.3	0.0	0.0	0.0
	0.09	0.0	6.0	1.6	1.7	1.0****	0.0	3.0
	0.1	0.0	6.0	0.7	1.3	0.0	0.0	1.0
N-doped CNTs	Control	0.0	1.0	0.2	0.4	0.0	0.0	0.0
	0.08	0.0	10.0	2.8	3.1	2.0****	0.0	5.0
	0.09	0.0	7.0	2.1	1.9	2.0****	0.0	3.0
	0.1	0.0	8.0	2.3	2.1	2.0****	0.0	4.0

p-value of pristine CNTs < 0.0001, p-value of N-doped CNTs < 0.0001

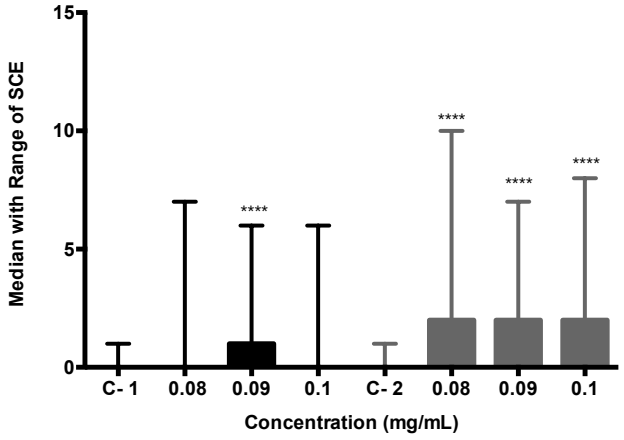

Figure 11. Sister chromatid exchange in human lymphocytes exposed to pristine CNTs (black bars) p<0.0001 and N-doped CNTs p<0.0001 (grey bars). C-1 and C-2 correspond to negative controls (RPMI-1640) and mitomycin-c [10μM] was used a positive control.

7.4.3 Comet Assay on HUAEC and HepG2

Figure 12 corresponds to the median and interquartile ranges of the comet tail length (μm) of HUAEC and HepG2 cells exposed to three sub–lethal concentrations of pristine and N-doped CNTs. There was a significant difference in the comet tail lengths measurements for the two types of cells exposed to the three sub-lethal concentrations of both types of CNTs. In general, there was greater variability in the comet tail length of cells treated with N-doped CNTs compared to cells treated with pristine CNTs. On the other hand, when we focus our attention on the type of cell used, it can be observed that HepG2 cells treated with N-doped CNTs show greater variability than HUAEC (Figures 12a and 12b).

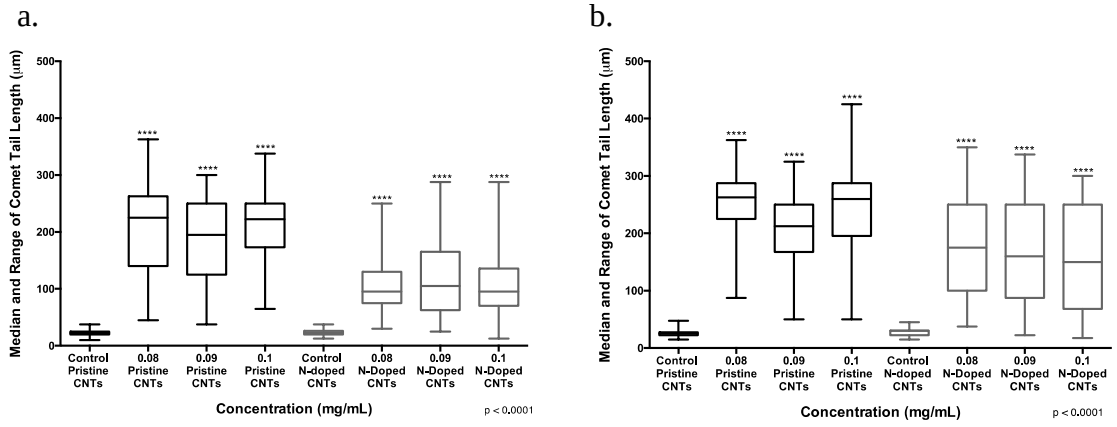

Figure 12. Comet tail length of a) HUAEC and b) HepG2 cells exposed to sub-lethal concentrations of pristine and N-doped CNTs during 24h. Cell culture media was used as the negative control and H_2O_2 [50μM] was used as a positive control.

Frequency of damaged cells was another parameter considered when accessing amount of DNA damage. Figure 13 shows the percentage of damaged cells according to the previously stablished damage levels (Table 1). The highest percentage of damaged cells are present in the total damage category. Figure 13a corresponds to HepG2 cells while figure 13b corresponds to HUAEC. It is evident that both types of CNTs damage the DNA of the cells, but the percentage of damage cells decrease as the concentrations of N-doped CNTs augment. Although the decrease in percentage of damage cells is not drastic, it is still evident and it is observed on both HUAEC and HepG2 cells.

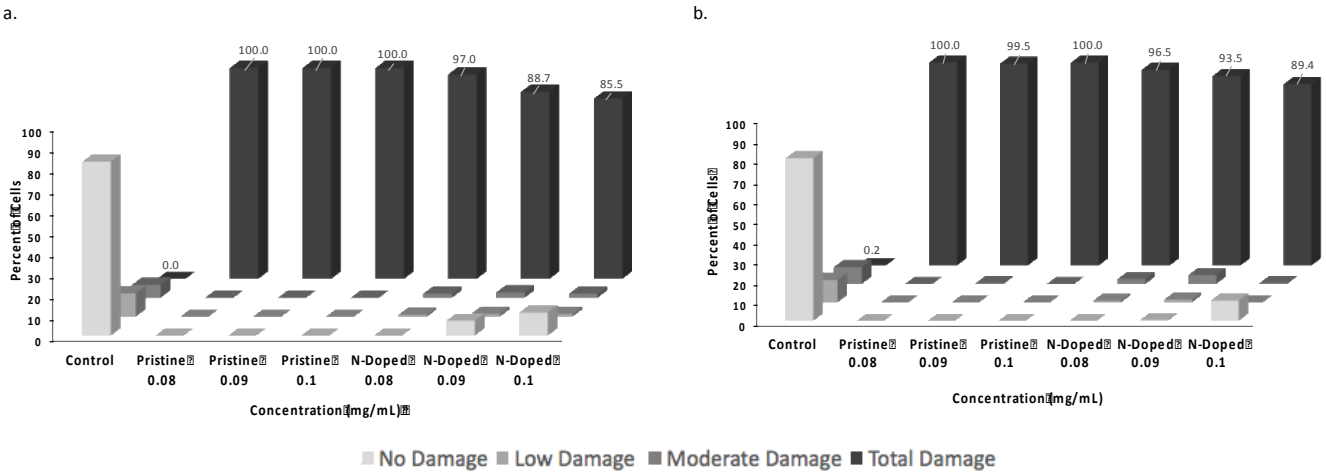

Figure 13. Percentage of damage levels in a) HepG2 and b) HUAEC cells exposed to sub-lethal concentrations of pristine and N-doped CNTs during 24h. DNA damage was assessed with SCGE. Cell culture media was used as negative control and H_2O_2 [50μM] was used as a positive control. Significant difference was found between length tail of the damaged cells and negative control p-value < 0.0001.

7.5 Life Cycle Dynamics

7.5.1 Cell Cycle Analysis by Flow Cytometry on HUAEC

A depiction of the cell cycle dynamics of HUAEC exposed to all ten concentrations (0.01-0.1mg/mL) of N-doped and pristine CNTs can be observed in figure 14. Changes in the cell cycle dynamics are represented by DNA quantitation distribution of cells in each phase of the cell cycle (G1/G0, S and G2/M). The lower segment of the bars represents cells in G1/G0, middle segment corresponds to cells in S and top segment corresponds to G2/M cells. There was no statistical difference when data obtained for each phase of the cell cycle were compared to the negative control ($p>0.9999$).

7.5.2 Cell cycle Analysis by Mitosis Inhibition in HepG2

Cell cycle dynamics of HepG2 by different inhibition times of mitosis with colcemid is represented in figure 15. The cell cycle duration was approximately between 13 and 16 hours for non-treated HepG2 cells (figure 15a), for cells treated with 0.1mg/mL of pristine CNTs the cycle duration was approximately between 14 and 15 hours (figure 15b), and cells treated with 0.1mg/mL of N-doped CNTs had a cell cycle duration between 12 and 15 hours approximately (figure 15c).

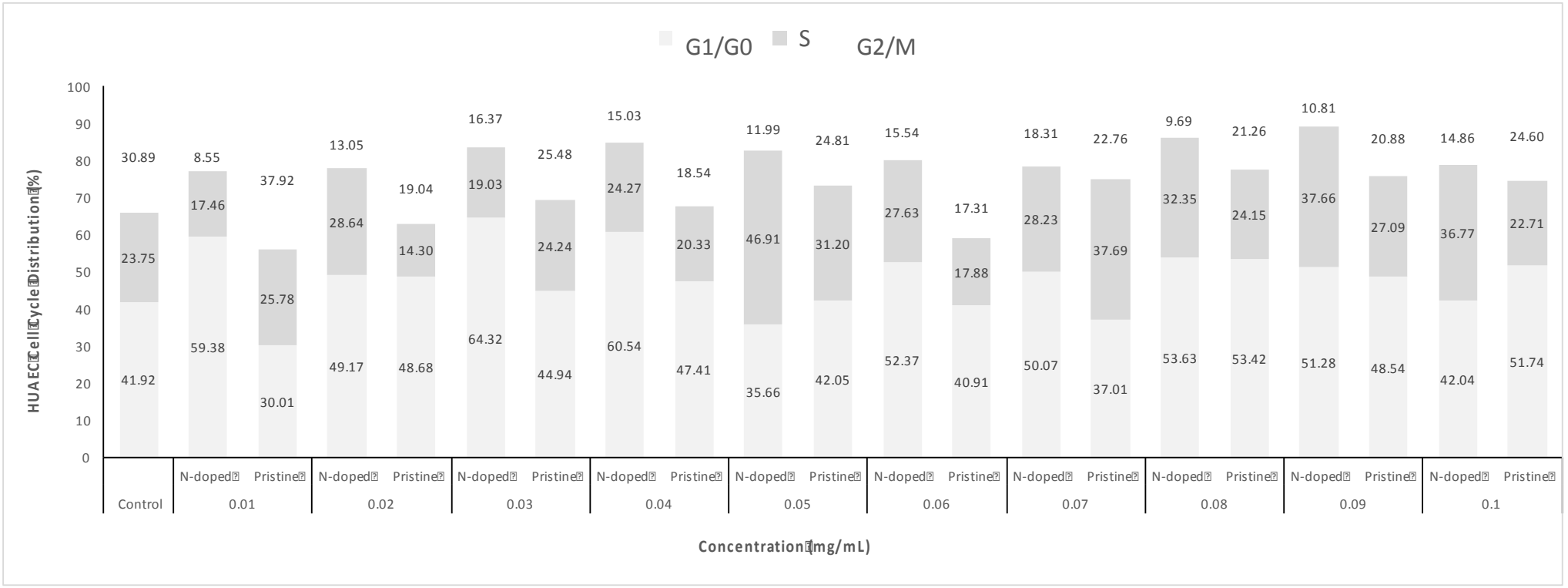

Figure 14. Cell cycle analysis by flow cytometry of HUAEC. Cells were exposed to different concentrations of pristine and N-doped CNTs during 24h. Percentage of cell frequency was determined for each phase of the cell cycle; phases appear in the graph from bottom up as: G1 (light grey), S (medium grey) and G2/M (dark grey), $p > 0.9999$.

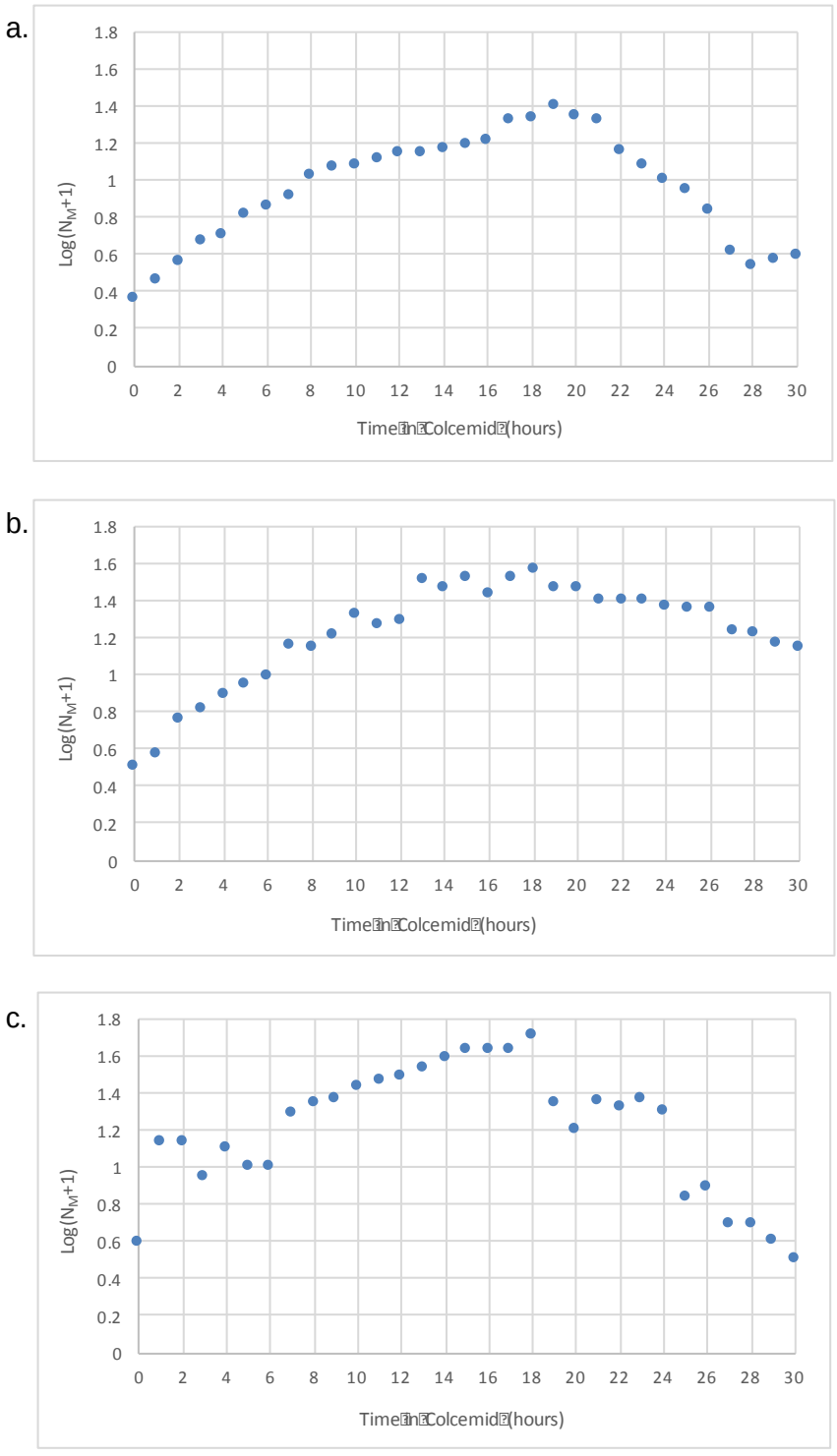

Figure 15. Life cycle dynamics of HepG2 cells by accumulation function of mitotic cells treated with Colcemid [10ug/mL]. Cells were monitored every hour up to thirty hours by calculating the mitotic index. a) plot of the accumulation function of untreated HepG2, b) plot of HepG2 treated with pristine CNTs [0.1mg/mL], and c) plot of HepG2 treated with N-doped CNTs [0.1mg/mL].

7.6 Mutagenicity Assay

In order to choose the three sub-lethal concentrations of both types of CNTs to perform the mutagenicity assay using strain TA98, a viability test was done using a range of concentrations from 10 to 0.1mg/mL. The viability test results with *S. thyphimurium* showed no reduction in the number of revertant colonies compared to the non-treated control nor change in the auxotrophic background (background lawn) therefore none of the concentrations evaluated caused any changes on the bacteria viability (data not shown). Based on these results, three sub-lethal concentrations [0.08, 0.09 and 0.1mg/mL] were used to perform the AMES test.

The average of revertant colonies and the mutagenic indexes of strain TA98 exposed to both types of CNTs in the presence and absence of S9 are reported in table 5. There was no evidence of mutagenic effect (frameshift mechanism) for bacteria treated with pristine CNTs, $p > 0.9$ in the presence or absence of S9. The average of revertant colonies for bacteria treated with N-doped CNTs was below the average of revertant colonies of the negative control (21 revertant colonies) for all concentrations except for the highest concentration (0.1mg/mL), which had an equal or lower number of revertant colonies as the control in the absence of S9. Mutagenic indexes of strain TA98 treated with three sub-lethal concentrations of both types of CNTs with and without metabolic activation are better visualized in the graph shown in figure 16. All mutagenic indexes were ≤ 1 which is evidence that both types of CNTs were not mutagenic.

Table 5 Average of reverse mutant colonies and mutagenicity index (MI) obtained after treating strain TA98 with N-doped and pristine CNTs.

Presence of S9	Compound (mg/mL)	Pristine CNTs			N-Doped CNTs		
		Mean	SD	MI	Mean	SD	MI
Without S9	Control	20	1	1.00	21	2	1.00
	0.08	16	7	0.80	18	6	0.85
	0.09	18	5	0.91	16	4	0.78
	0.1	20	6	1.01	21	8	0.99
With S9	Control	23	3	1.00	23	2	1.00
	0.08	23	11	1.00	13*	3	0.56
	0.09	20	7	0.88	15*	6	0.66
	0.1	24	10	1.04	16*	3	0.68

Positive controls used for TA98 were Daunomycin [6µg/plate], Mitomycin C [0.5µg/plate] in absence of S9, 2-Aminoanthracene [1-5µg/plate] as a positive control for metabolic activation, and PBS was used as a negative control. When compared to the negative control, N-doped CNTs in the presence of S9 had p-values of 0.006 and 0.01 for [0.08mg/mL] and [0.09 and 0.1mg/mL] respectively.

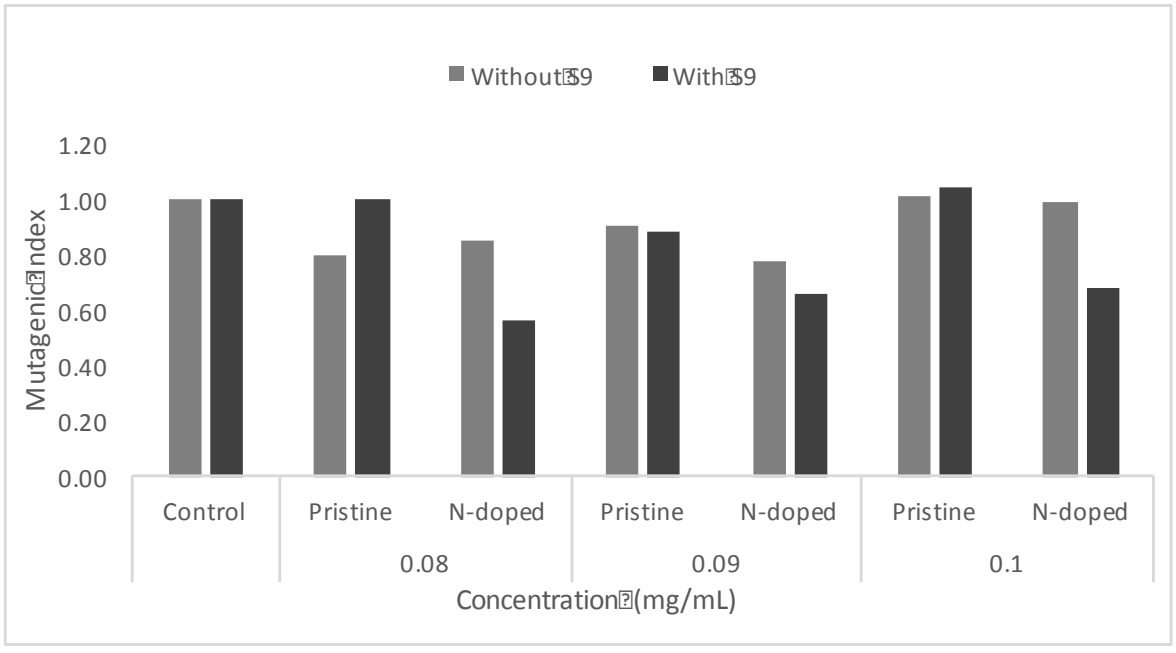

Figure 16. Mutagenic Index of strain TA98 treated with both types of CNTs in the presence and absence of S9, negative control used PBS.

8. Discussion

The general objective of this research was to evaluate the cytotoxic, genotoxic, and mutagenic effect of two types of CNTs on human cells. Carbon nanotubes used in this

research were dispersed by ultra-sonication in cell culture media as it can be seen in figures 3b and 3c. Ultra-sonication parameters such as amplitude, frequency and sonication time chosen to disperse the CNTs samples were selected based on data reported by Hielscher et al. [103]. Mechanical dispersion methods such as ultra-sonication are used to separate CNT agglomerates, but this method also compromises the length and layers of CNTs, resulting in fragmentation as well as layer shedding. Other authors such as Hilding et al. [62] investigated the effects of sonication on the morphology of CNTs and reported that mechanical dispersion methods alter the length and diameter of CNTs. Based on the data reported by the previous two authors, and visible changes observed in figure 3 it could be assured that CNTs employed in this research were successfully dispersed by ultra-sonication. Dispersion of CNTs in aqueous solutions has also been achieved by other researchers [104]–[108], but the process differs in the use of surfactant agents, which stabilize the solution and prevent aggregation of the CNT particles. Murdock et al. [109] also prepared CNT dispersions prior to *in vitro* exposure of cells, the authors used water as the dispersion liquid as well as cell culture media and serum. It is worth to mention that RPMI-1640 supplemented with 5% fetal bovine serum was one of the first dispersion liquids used to sonicate CNT samples employed in this research, but the attempt resulted in failure due to excessive foaming of the dispersion liquid caused by the sonicator wand as well as contamination of the sample. Therefore, dispersion of the CNT samples was finally achieve in RPMI-1640 without FBS and by using an ultrasonic wand.

Our results indicate that cell viability evaluation by trypan blue exclusion test of HUAEC and HepG2 were not significantly affected by any of the two types of CNTs, at the ten concentrations evaluated; although the concentration influence cell viability, all viability results remained above 80%. Cell viability results of HUAEC obtained by flow cytometry using PI and $DIOC_6$ are congruent with results obtained with the trypan blue exclusion test. No significant changes in cell viability were detected for the two types of CNTs at any of the tested concentrations. CNTs were well dispersed and the sonication process substantially reduced their length and diameter (figures 3b and 3c) allowing the CNTs to penetrate the cell membrane by diffusion or enter via membrane pores [44]. Both techniques used to evaluate cell viability: trypan blue exclusion test which evaluates cell

membrane integrity, and cell viability by flow cytometry which also evaluates mitochondrial activity corroborate these findings. It is possible that once CNTs got inside the cell they remained dispersed or did not form large agglomerates, which cause cell viability to decrease as reported by Wick et al. [110].

Other authors have reported that presence of large agglomerates inside the cell cause a decrease in cell viability due to accumulation in lysosomes which cause injury to the membrane. The cell viability results obtained in this research remained above 80%, nevertheless, many factors such as the type of cell tested, CNT synthesis, physical characteristics (size, length, shape, amount of carbon layers) and surface structure produce a negative impact on cell viability [30], [50], [51], [55]–[57], [105]. One particular research conducted by Flahaut et al.[112] reported that cell viability of HUAEC decreases due to an increment in CNT surface area (non-agglomerated CNTs) present at low concentration. Although Flahaut et al. found a dose dependent viability response, our research with HUAEC did not exhibit the same effect, in fact our results did not show a decrease in cell viability related to dose. On the other hand, a significant difference on the cell viability evaluated by flow cytometry of HepG2 exposed to N-doped CNTs was detected for cells exposed to 0.03mg/mL of N-doped CNTs. In figure 9c the a p-value of 0.0423 for PI+ cells was reported, the slight difference is observed at concentration 0.03mg/mL of HepG2 exposed to N-doped CNTs, and the difference is better visualized in figure 10b; these results agree with results reported by Flahaut et al.

Cell biomarkers assays (CA and SCGE) evidenced DNA rupture (Figures 12 & 13) and chromatid and chromosomal breaks (Tables 2 and 3) by production of highly intrinsic reactive oxygen species (ROS) induced by CNTs. The main ROS induced by CNTs are superoxide anions and hydroxyl radicals which are the primary DNA damaging species [44], [53]. Indirect damage to the cell by production of ROS could be generated by traces of catalyzer encrusted in CNTs, in this case iron and cobalt were used as catalyzers and could be directly linked to the generation ROS via the Fenton reaction [45]. The CNT surface area which is related to the graphene structure could contain carbon centered radicals, and structural defects in the CNT lattice could have formed during their growth

and purification process [46], therefore , DNA damage can also be attributed by the direct interaction of CNTs with structural these defects generating vulnerable sites that can interact with macromolecules such as proteins, lipids and DNA [113],[114]. Finally, the innate response of the cell to CNTs could also augment the production of ROS. CNTs can act as a double edge sword because they can deplete the amount of antioxidants present in the cell and therefore generate DNA damage or the structural defects present in CNTs can act as scavengers of ROS rather than increase their production [115], [116].

The chromosomal aberration test performed on human lymphocytes exposed to three sub-lethal concentrations of pristine CNTs showed that there was a significant difference according to the p-values of 0.011 and 0.001 corresponding to chromatid and chromosomal breaks, respectively. The highest concentration of pristine CNTs induced the most chromatid (6.5%) and chromosomal (5.5%) damage, although the percentage of chromosomal damage remained below 10% these damages are still detrimental to the cell. Since the cell has a better chance of repairing single strand breaks while double strand breaks are more difficult to repair [46] and could become mutations given that these affected cells did not perish but instead they continue through the cell cycle (Figures 8, 9, 14 and 15).

Chromosomal aberrations were also evaluated in HepG2 cells, with both types of CNTs and the three sub-lethal concentrations (0.08, 0.09 and 0.1mg/mL). The results obtained for this assay were not significantly different when compared to the negative control $p>0.05$ (Table 3). The main pathway of DNA damage by CNTs is the formation of ROS, in this case it is possible that CNTs did not induce the production of ROS possibly due to the drug metabolizing enzymes present in this type of cell. Compared to this research, other authors have reported cytotoxic and genotoxic effects obtained with HepG2 exposed to SWCNTs at concentrations ranging from 5-50µg/mL [117].

The sister chromatid exchange assay provides information about the cell's ability to repair damaged DNA, therefore the presence of sister chromatid exchange reflects an existing lesion on the DNA. The SCE results obtained for cells treated with the sub-lethal

concentrations of N-doped CNTs showed a highly significant difference in the percentage of SCE when compared to the negative control $p<0.0001$, while pristine CNT treated cells were significantly affected by only one of the concentrations evaluated (0.09mg/mL) when compared to the negative control, $p<0.001$ (Figure 11). A possible explanation in the increase in SCE in human lymphocytes exposed to N-doped CNTs could be that CNTs induce the formation of DNA exchange because they interact with DNA by forming adducts [81]. Adducts could generate in two ways: the entire CNT attaches to de DNA or any structural defected regions on the CNT interact with the genetic material [118]. It is worth to note that N-doped CNTs did not induce any type of chromosomal alterations on human lymphocytes at the evaluated concentrations $p>0.05$ (Table 2). In order to test this hypothesis, it would be necessary to perform other test with the purpose of identifying the CNT adduct that binds to the DNA. In contrast, pristine CNTs yield an increase in the percentage of SCE in human lymphocytes only for 0.09mg/mL, this same concentration did not induce chromosomal breaks, but only induced 1% of chromatid breaks. Pristine CNTs induced the formation of SCE in a less frequent manner compared to N-doped CNTs, possibly by the same mechanism (adduct formation). N-doped CNTs interact more frequently with the DNA due to the incorporation of nitrogen which increases their DNA affinity [119], therefore it is possible that N-doped CNTs augment the formation of adducts with the DNA, which are evidenced by an increase of SCE (Figure 11).

The presence of chromatid damage in human lymphocytes exposed to pristine CNTs [0.09mg/mL] could be reflected in the SCE frequency found in human lymphocytes exposed to this same concentration (Table 4 and Figure 11). As previously mentioned, DNA damage could be different depending on the type of cell used, for example van Berlo et al. [47] and Szendi et al. [121] did not find SCE with CNTs in other cell lines (CHO A88 and A549 cells) different to human lymphocytes in the presence or absence of metabolic activation. It is important to note that DNA damage with the proposed biomarkers in HUAEC was only achieved by the comet assay, CA and SCE with HUAEC could not be achieved due to the susceptibility of this cells to conventional anti-mitotic agents (colcemid and colchicine), which prevented from obtaining chromosomal extends due to cell death a few minutes after exposure to the anti-mitotic agent. Unfortunately, no information was

found on the use of antimitotic agents such as colchicine and colcemid on HUAEC on all the reviewed literature, nevertheless several tests were performed to minimize the toxicity of anti-mitotic agents on HUAEC without success.

There were not any alterations on the cell cycle dynamics of HUAEC exposed to both types of CNTs at concentrations ranging from 0.01 to 0.1mg/mL when compared to the negative control (Figure 14), which means that cells were not arrested at any of the cell cycle phases (G1/G0, S and G2/M). Cell viability of HUAEC was not altered but there was a highly significant difference in the comet length tail (Figure 13) and more than 65% of cells were categorized as totally damaged (Figure 12). Results obtained from the cell cycle dynamics test and the comet assay show that although the cell's DNA is highly damaged it continues through the cell cycle, which is dangerous because mutations become fixed, accumulated and develop into cancer. Similarly, Ursini et al. [81] also reported simultaneous, direct and oxidative damage to de DNA evaluated by formamido–pyrimidine glycosylase (fpg) comet test in human epithelial lung cells treated with MWCNTs.

Cell cycle dynamics of HepG2 measured by accumulation function with colcemid in mitosis treated with both types of CNTs with the highest concentration (0.1mg/mL) only showed that the duration of the cell cycle of cells treated with pristine and N-doped CNTs is similar to the duration of the cell cycle of untreated HepG2 cells, meaning that the cells are not being arrested at any of the cell cycle phases with similar biological consequences mentioned for HUAEC at the same concentration. The cell cycle arrest results obtained for this research differed from results reported by other authors [121], which concluded that cells are arrested in G1 phase by reducing the proliferation rate in C6 rat ganglioma cells exposed to MWCNT during 24 hours at concentrations ranging from 0-400µg/mL.

This research showed that CNTs did not induce mutagenic changes in *S. typhimurium* TA98 with the three sub-lethal concentrations evaluated in presence or absence of the metabolic activator (S9), which means that metabolites produced by the metabolic oxidation system (P450) do not induce mutagenicity effect. Despite the fact that the number of revertant colonies of strain TA98 treated with N-doped CNTs was significantly

less than the revertant colonies of the negative control it cannot be concluded that there was a protective effect of CNTs because there was not a dose dependent response (Table 5) [120], [122]. On the contrary, if CNTs had a protective effect the number of revertant colonies would be dose dependent, with lower number of revertants in response to high concentrations of CNTs. Similar results were obtained by Di Sotto et al. [122] and Szendi et al. [120] which concluded that there was no mutagenic activity in strains TA98 and TA100 exposed to MWCNTs.

The lack of mutagenicity found in this research with TA98 can be attributed to generation of ROS induced by CNTs which is not detected by this strain. It is suggested to evaluate the mutagenicity effect with a different strain such as TA102 which detects mutations caused by ROS.

9. Concluding Remarks and Perspectives

In general, both types of CNTs presented a genotoxic effect in HUAEC, HepG2 and human lymphocytes, with no significant effect in the viability and cell cycle dynamics, which implies a high risk on the genetic material of all organisms, exposed to CNTs, because mutations could be passed along future generations. Since the mutagenicity results obtained with *S. thyphimurium* indicated that the CNTs did not cause mutations, future research could be to evaluate the mutagenicity in eukaryotes, since a possible explanation for the lack of mutagenicity in prokaryotes is due to the lack of penetration of the CNTs inside the bacteria.

The results obtained for this research as well as results reported by other authors are extremely variable, such variability is attributed to the physico-chemical characteristics of CNTs such as surface area, size, length, structure, shape, traces of catalyzer, mode of dispersion, functionalization/doping and amount of carbon layers (single or multi-walled). These characteristics imply that multiple evaluations as well as implementation of different biomarkers are required before incorporating CNTs in any medical devise. Choosing the correct biomarker is crucial to test CNT effects, since some physical and chemical

characteristics of CNTs like the ones previously mentioned could interact with products of the chosen test by altering the result and generating false positive results.

Other aspects to consider when evaluating CNTs would be the target cell, tissue or organism because the effect exerted by CNTs also depends on the permeability of the cell, the type of cell/tissue and its response mechanism, therefore different and variable results are obtained, eventually generating erroneous results and leading to the need of choosing the right bio-system where CNTs will be tested. As well as to evaluate the effect of CNTs with other biomarkers, different from the ones used here, with the purpose of identifying other types of damage.

For other future research, it would be imperative to perform compatibility tests using concentrations lower than 0.05mg/mL since it was observed that CNTs affect the cell cycle as well as the cell viability at lower concentrations. Finally, there is not a lot of information available about the bioaccumulation of CNTs on cells and organs; therefore, it would be of great impact to research the mechanism of elimination of CNTs by cells and organisms.

10. References

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