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Atherosclerosis, a prevalent contributor to cardiovascular disease (CVD) on a global scale, is primarily triggered by inflammation, which plays a critical role in initiating the disease process. This inflammatory response leads to endothelial cell dysfunction or damage, ultimately resulting in the formation of plaque buildup within the inner walls of arteries. The emerging nanomaterials provide new prospects to lower the economic and healthcare costs associated with CVD. Carbon nanodots (CNDs), a type of nanoparticle, are particularly attractive due to their biocompatibility, fluorescent capabilities, and potential antioxidant properties. While much research has been conducted on the use of CNDs as bioimaging and drug-delivery tools, their potential anti-inflammatory effects, particularly in the cardiovascular system, have yet to receive much attention. The aorta is particularly vulnerable to atherosclerosis, being the largest affected area. In this study, HAEC (human aortic endothelial cells) were chosen as the cell line due to their capability to express endothelial cell surface biomarkers, and their common use in vascular research has provided a comprehensive understanding of cell lines. However, the impact of CNDs on HAEC has not been investigated yet. In this study, the impact of CNDs on TNF-α induced inflammation in HAEC was studied. Our results demonstrate CNDs inhibited the production of inflammatory genes, such as IL-8, E-Selectin, and CCL2, in vitro in response to TNF-α. With concentrations of up to 0.6 mg/mL used, CNDs did not show any cytotoxic capabilities in our results in HAEC. Fluorescence microscopy data indicated that HAEC were able to uptake CNDs at the concentrations used. The NF-κB Luciferase Reporter Cell assay results showed that CNDs have the ability to reduce TNF-α-mediated increase in NF-kB activity. The results of the Nrf2 pathways also indicated that CNDs can activate Nrf2 transcription, thus

leading to an increase in Nrf2-mediated upregulation of various antioxidant genes, including HO-1, GCLC, NQO-1, and GR. Through these results, it can be suggested that the anti-inflammatory effects of CNDs can be related to the downregulation of the NF-κB pathway and the up-regulation of the Nrf2 pathway signaling. This is the first study to examine the effects of CNDs on human aorta endothelial inflammation.

A STUDY OF THE EFFECT OF CARBON NANODOTS ON TNF-α INDUCED HUMAN AORTIC ENDOTHELIAL INFLAMMATION

by

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Approved by

Dr. Zhenquan Jia
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DEDICATION

I would like to dedicate this thesis to my support system. This includes my amazing parents for pushing me to work hard and supporting me through countless sleepless nights. My friends, for constantly pushing me to keep going and supporting me every step of the way. None of this would have been possible without Allah (SWT) blessings.

APPROVAL PAGE

This thesis written by Urooj Amin has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I: INTRODUCTION

Cardiovascular Disease: Global Impact

Cardiovascular disease (CVD) has a substantial impact throughout the world. Due to these devastating effects, CVD has become a significant focus in research. In the United States, a quarter of all American deaths are attributed to CVD. Projections show that in less than 20 years, there will be a large increase in medical costs and in the overall population with CVD markers, which is the main factor for the push to improve current treatment [1]. Atherosclerosis (CVD) can be categorized as ischemic heart disease, cerebrovascular disease, or coronary artery disease, depending on the location [2]. Inflammation is one of the most critical steps in the initiation process of atherosclerosis. This step also leads to cellular dysfunction or damage in the endothelial cell lining, leading to plaque buildup formation in the inner arterial walls [3]. Many previous studies demonstrated that proinflammatory mediator tumor necrosis factor-α (TNF-α) is a critical regulator of atherosclerosis, along with cell adhesion molecules and chemokines that are secreted by damaged endothelial cells [4-6]. Some of these cell adhesion molecules and cytokines include monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), interleukin (IL)-8, interleukin 1 β (IL-β), C-C motif chemokine ligand 2 (CCL2), and E-Selectin [4-7]. Reactive Oxygen Species (ROS) and oxidative stress play a critical role in the progression of endothelial damage through the expression of multiple proinflammatory adhesion molecules and chemokines [5, 8]. Nonetheless, the underlying causes of CVD are not fully understood, and the ramifications of the development of atherosclerosis need to be discovered further.

ROS and Nrf2-Mediated ROS Detoxification Molecules Associated with Atherosclerosis

ROS formation can occur through different cellular processes that occur within cells that produce molecules that contain oxygen and have reactive capabilities [8, 9]. ROS are described as mediators of inflammatory responses and have the ability to activate cell signaling and increase the production and release of proinflammatory cytokines, which are all factors that lead to inflammatory responses [10]. Non-free radicals such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) have longer half-life as compared to free radicals such as superoxide anions ($O^{2-}\bullet$) and hydroxyl radicals ($OH\bullet$) which have more reactive capability [8, 9]. The lower reactivity rate of H_2O_2 allows molecules to diffuse through the cell membrane while being able to travel further and therefore resulting in a critical signaling molecule [9, 11]. However, when there is an overproduction of ROS occurring, it leads to damage in DNA, lipids, and proteins, which ultimately leads to cell apoptosis, necrosis, and death as a result of the high reactivity of the oxygen-based molecules.

The cells contain an antioxidant/anti-inflammatory defense system. When ROS levels significantly exceed the defense system's capacity, oxidative stress occurs. The action of the transcription factor Nrf2 has been shown to increase the expression of many antioxidant/anti-inflammatory enzymes, thereby reducing ROS [12]. Overall, Nrf2 is a major and critical regulator for antioxidative/anti-inflammatory enzymes, cellular detoxification, and cytoprotective genes [13, 14]. Under typical conditions in the body, Nrf2 is actively produced in the cytoplasm of the cells. The ability to maintain low levels of free Nrf2 is associated with the Keap-1 repressor protein [13]. When a stressor is introduced into the body, such as free radicals, Nrf2 translocates to the cell's nucleus, where an accumulation can occur [15]. This process leads to the production of antioxidant proteins through the binding of Nrf2 to antioxidant response

elements. Nrf2 and Keap-1 systems can be seen as an anti-stress mechanisms with the goal of preserving cellular homeostasis [16]. The dysregulation of the Nrf2 pathway is associated with various diseases and inflammatory conditions, such as atherosclerosis [14, 17, 18]. Therefore, Nrf2 can be a prospective therapeutic target for the treatment of inflammation-mediated atherosclerosis.

The Role of Nuclear Factor Kappa B (NF-κB) Pathway in Inflammation

There have been comprehensive studies that describe the properties of NF-κB and indicate that the activation of NF-κB is critical in leukocyte adhesion to the endothelium through the transcription regulation of chemotactic cytokines and vascular adhesion molecules [19-21]. In patients, further studies showed that the activation of NF-κB has been found to be associated with the pathogenesis of atherosclerosis [22, 23]. TNF-α has been described to be a potent activator of NF-κB. The transcription factor for NF-κB is found in the cytoplasm of the cell, but it remains inactive due to I-κB, an inhibitor, being bound [24]. When this inhibitor is bound, the p50/65 heterodimer of NF-κB cannot enter the cell's nucleus [20, 21]. Through phosphorylation and ubiquitination of the inhibitor, NF-κB p50/65 heterodimer can enter and travel through the cell and bind with promoter regions. Once it attaches, there is an effect that causes an increased production of cytokines, chemokines, and adhesion molecules. This production stimulation signals the immune system to respond [19-21].

Carbon Nanodots and its Role in Cardiovascular Diseases

Searching for more prominent alternative ways to treat oxidative and inflammatory stress has important clinical implications for atherosclerosis patients due to current treatments' limitations. There have been several advances in the biomedical community regarding nanomedicine. Using nanoparticles as the treatment for many ailments has been a significant focus of research. Carbon nanodots (CNDs) are extremely small particles that can be less than 10 nm in size and have been used in nanotechnology to be modified for specific and unique uses for studies [25]. The synthesis of CNDs is beneficial due to its simplicity and affordability, and the materials used to construct the nanoparticles can modify the properties. There are two main methods of CNDs synthesis, which include top-down syntheses and bottom-up syntheses [25, 26]. Both categories contain multiple approaches and techniques to synthesize CNDs, with oxidation as a common factor. In top-down syntheses, the bulk carbon material is broken down into powder form, which is then transformed into ultra-small sizes and clustered together to form carbon dots [27]. There are various techniques to achieve this, some including arc-discharge, laser ablation, and chemical oxidation, each with its advantages. Through top-down syntheses, carbon dot sizes are below 10 nm, they have a spherical or sheet shape, and the height is below 3 nm [27, 28]. The bottom-up syntheses are developed to produce CNDs from organic molecules. This method involves taking precursor molecules from small organic molecules and ionizing, dissociating, evaporating, or sublimating these molecules to form ions, radicals, and electrons. These are then condensed to form carbon dots. This synthesis approach also has numerous techniques to achieve carbon dots, and the size is below 10 nm.

CNDs' extremely small size and conjugating and doping capabilities allow the dyes and stains to penetrate the cell membrane and the nucleus easily [29]. CNDs have been used to

bioimage cancer and immune cells due to these properties. Due to CNDs' photoluminescent properties, bioimaging, as well as biosensing, is possible. These two areas have been extensively researched and explored, but researchers are also discovering the potential mechanisms for drug delivery with CNDs [30]. Drug delivery is a biomedical application that has become more prevalently studied due to CNDs biocompatibility [31-33]. One of the techniques studied has included synthesizing CNDs with certain drugs to provide treatment for diseases [33]. Among them, aspirin-based and Metronidazole-based CNDs have been synthesized with the goal of delivering pharmaceuticals directly to the cells in lower doses than typically needed to be effective [31]. There has been extensive research looking at drug delivery, bioimaging, and biosensing, but further research is needed to understand the full capabilities and potential underlying mechanisms.

It has been previously suggested that CNDs are potent antioxidants and detectors for ROS and have protection against it and oxidative stress [34]. Though the mechanisms behind these antioxidant properties remain unclear, the oxidant-free radical scavenging proficiencies are thought to relate to the electron donor capabilities of CNDs. A large portion of the surface area of CNDs is made up of carboxyl and amino functional groups [28]. These are thought to be responsible for the donation and acceptance of electrons. Recent studies have shown that along with the modification of these two groups, the antioxidant properties of CNDs for scavenging DPPH free radical molecules also increase [35]. This has been linked to significant potential to facilitate future biomedical application purposes [30].

There has been extremely limited exploration of the impact of CNDs' antioxidant properties on the cardiovascular system. In a recent study conducted in my lab, the direct ROS scavenging abilities of CNDs were examined [36]. In this study, electron paramagnetic

resonance (EPR) spectroscopy was used to view the generation of hydroxyl radicals. The addition of 0.01 to 0.3 mg/mL of CNDs showed a significant dose-dependent increase [36]. oxLDL is a biomarker for inflammation and atherosclerosis. Our study further demonstrated the anti-inflammatory effects of CNDs against oxidized low-density lipoprotein (ox-LDL) induced endothelial dysfunction by looking at mediated monocyte adhesion to human dermal microvascular endothelial cells (HMEC-1) [36]. Also, our lab examined the cellular uptake of CNDs and their effect on TNF-α-induced inflammation in HMEC-1 cells [37]. HMEC-1 cells were treated with 10 ng/mL of TNF-α and 0.03-0.3 mg/mL of CNDs. Through qRT-PCR, it was seen that there was a significant decrease in TNF-α-induced IL-8, IL1-β, ICAM gene expression in a dose-dependent manner in the presence of CNDs. CNDs can also be uptake by HMEC-1 via the endocytosis pathway [37].

When a thickening or hardening of the arteries occurs because of plaque buildup in the lining of the arteries, this is known as atherosclerosis. This disease is widespread, affecting the majority of the arteries in the body, including those in the heart, brain, pelvis, kidneys, arms, and legs. With the aorta being the largest artery in the body, this is one of the largest affected locations in atherosclerosis. The aorta's function is to distribute oxygenated blood from the heart to all the organs, and with this critical responsibility, it is important to understand. Primary human aortic endothelial cells (HAEC) are directly isolated from the human aorta. These cells create the inner lining of the aorta. HAEC play a crucial role in cellular adhesion molecule expression, inflammatory response regulation, and vascular homeostasis [38, 39]. An early marker of atherosclerosis is damage to or dysfunction of HAEC [38, 39]. These cells have been used widely as a model in vascular research due to these properties. To study arterial disease, inflammation, and atherosclerosis, these cells have been used because of their ability to be used

for adhesion assays with several human monocytes. HAEC can express endothelial cell surface biomarkers associated with regulating inflammatory responses and have LDL functional uptake properties [38, 39]. These biomarkers include vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E-selectin [40]. When the catalytic subunit of human telomerase reverse transcriptase (hTERT) is encoded in primary HAEC, this leads to an extended lifespan of primary endothelial cells without any cellular transformation or genomic incompatibility [39, 41]. This allows HAEC to demonstrate a breakthrough in vascular research through this transformation while maintaining essential characteristics. In our lab, previously, the effects of CNDs have been observed on HMEC-1 cells, but it is unknown how CNDs can affect HAEC cells which leads to my aim to study this further. Specifically, tert-HAEC cells have been chosen for my study due to their ability to show adhesion molecule characteristics, endothelial markers, and aortic endothelial origin. The aim of this study was to examine the effects of CNDs on TNF-α induced inflammation in human aorta endothelial cells.

CHAPTER II: METHODS AND MATERIALS

Cell Culture

Complete medium was produced with ATCC Vascular Cell Basal Medium and an Endothelial cell growth kit. HAEC cells were cultured in ATCC Vascular Cell Basal Medium with 10 ng/mL Endothelial Growth Factor (EGF), 1 μg/mL hydrocortisone, 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. Placed in incubators set to 37C and 5% CO2, cells were grown in Cellstar® Filtered Cap 75 cm2 cell-culture treated, filter screw cap flasks, while media was changed every 2-3 days, maintaining splitting into the new passage at 85-90% confluence.

CNDs Synthesis

CNDs were synthesized using 0.96 g citric acid, 1 mL ethylenediamine, and 1 mL deionized water, mixed in glass vials. The mixture was then heated in a microwave reactor at 300W for 18 minutes. To create the brown solid, the temperature was controlled below 150C and diluted in 5 mL of deionized water. Through a dialysis membrane, this was dialyzed with MWCO of 100Da for 24 hours.

CNDs Characterization

Using the Cary® Eclipse TM Fluorescence Spectrophotometer, the absorbance and fluorescence properties of CNDs were looked at. CNDs were diluted to 0.01 mg/mL in DI-H2O. These were then measured for fluorescence in a quartz cuvette. Using a carbon 1s X-ray photoelectron spectroscopy (CPS, ESCALAB 250 Xi, Thermo Fischer), the surface chemistry of CNDs was characterized. To view and characterize the structure of the CNDs, a Fourier transform infrared spectroscopy (FTIR, varian670) was used. The size and morphology of the CNDs were characterized using atomic force microscopy (AFM).

CNDs Uptake Assay

HAEC cells were cultured to 85% confluency and treated with CNDs at 0, 0.1, 0.3 and 0.6 mg/mL concentrations for 1 hour in a 6-well Corning cell culture plate. The treatment was removed after 1 hour, and cells were washed with PBS to remove any unbound CND. To determine CNDs uptake, cells remained unfixed in the plate and media was removed from each well. Fluorescence was measured to determine the number of CNDs uptake from the cells. Images were taken of each well using phase contrast and fluorescence and the images were then overlayed to view cells and fluorescence. Image J was used to quantify the intensity of fluorescence.

Cell Viability with Trypan Blue

HAEC were grown in 6-well Corning cell-culture plates to 85-90% confluence. Cells were then washed two times with PBS and cells were treated with 0, 0.1 and 0.3 mg/mL of CNDs for 1 and 24 hours. Once treatment was completed, cells were washed two times with PBS. Cells were collected using Trypsin and Trypsin neutralizing solution and spun down in a centrifuge at 1,000 RCF for 10 minutes. 20 uL of cells and 20 uL of Corning Trypan Blue Solution (0.4%) was added. 10 uL of the cell-trypan blue mixture was pipetted into the hemocytometer and cell viability was measured.

FITC Nexin (Flow Cytometry)

For 1 and 24 hours, cells were treated with CNDs as described previously. In the absence of any treatment, negative control of cells in a complete growth medium were prepared. Once cells were harvested, they were washed with 1x PBS and centrifuged at 1,600 rcf for 7 minutes at 4°C. Cells were diluted out to 1x106 cells/mL with 1X Annexin Binding Buffer once PBS was decanted. 100 µL of the diluted cell sample was placed in 1.5 mL Eppendorf tube, along with 2.5

μL of FITC Annexin V and 1 μL of 7-AAD, and this mixture was gently vortexed. For 15 minutes, the mixture was incubated in the dark at room temperature (25°C), and following incubation, 400 μL of 1X annexin-binding buffer was added to the samples. Samples were mixed gently, kept on ice, and analyzed using Guava EasyCyte Cytometry System to differentiate between dead and living cells.

Nrf2 Luciferase Reporter Cell Line

The Nrf2 luciferase reporter cell line was obtained from Signosis, which was developed by co-transfecting the Nrf2/ARE luciferase reporter vector and hygromycin expression vector. HEK293 Human embryonic kidney cells were thawed and cultured in a complete growth medium. Cells were cultured in a white opaque 96-well plate until 90% confluence. Once confluence was reached, media was discarded, and cells received treatment. Nrf2 cells were treated with a concentration of CNDs for 24 hours. Once incubation was completed, media was removed. 20 μL of lysis buffer was added to each well and kept at room temperature for 15 minutes. Once completed, 100 μL of luciferase substrate was added to each well, and the plate was immediately read in the Bio-Tek Synergy™ plate reader.

NF-κB Luciferase Reporter Cell Line

NF-κB luciferase reporter cell lines were obtained from Signosis. HEK293 Human embryonic kidney cells were thawed and cultured in a complete growth medium. Cells were cultured in a white opaque 96-well plate until 90% confluence. Once confluence was reached, media was discarded, and cells received treatment. NFκB cells were co-treatment with varying concentrations of CNDs (0.1 and 0.3 mg/mL) and 10 ng/mL of TNF-α for 24 hours. Once incubation was completed, media was removed. 20 μL of lysis buffer was added to each well and

kept at room temperature for 15 minutes. Once completed, 100 µL of luciferase substrate was added to each well, and the plate was immediately read in the Bio-Tek Synergy™ plate reader.

RNA Extraction

RNA was extracted using a Zymo Research Direct-zol RNA miniprep kit according to the manufacturer's protocol with the following alterations. HAEC cells were cultured in 6-well Corning plates and treated as previously described. Equal parts TRIzol and ethanol were added to each well and then transferred to a Zymo-Spin IICR column and centrifuged. DNase I treatment was used to remove contaminating genomic DNA. A Direct-zol RNA PreWash was used, followed with an RNA wash buffer. RNA was eluted and purity and integrity was measured using Thermo Scient ific Nano Drop 2000/2000c Spectrophotometer. RNA was stored at -20 C and used for further assays.

cDNA Synthesis

HAEC cells were cultured, treated, and RNA extraction occurred as described previously. Using a Thermo Scientific™ Nanodrop 2000, extracted RNA was quantified and diluted to a 1000 ng/µL concentration. Diluted RNA was combined with 5 µL of 5X First-Strand Buffer, 1.25 µL of deoxynucleotide triphosphate (ddNTP) solution, 1.25 µL of Random Primer, 0.625 µL of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and 14.875 µL of DEPC H2O. This mixture will be converted to cDNA using an Applied Bioscience™ Veriti™ 96-Well Thermal Cycler.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

As described above, cells were treated, RNA extracted, and cDNA synthesized. The genes of interest were targeted using the cDNA by combining 10 µL of Power SYBR Green PCR Master Mix, 5 µL of DEPC H2O, 2 µL of each 5 µM forward and reverse primers for genes of

interest, and 1 μL of diluted cDNA (1:10). The genes of interest were CCL2, IL-1β, IL-8, and E-selectin with GAPDH as the housekeeping gene. The Applied Biosystems™ StepOnePlus™ Real-Time PCR system will be run for 40 cycles with each phase as 95°C for 15 seconds, a 58°C phase for 1 minute, and a 60°C for a 10-second phase. A comparative threefold cycle (Ct) value will be used to quantify gene expression. The Ct value is the point at which the machine detects the lowest amount of fluorescence, which only occurs once SYBR® Green attaches to the double-stranded DNA.

Animal Project (APOE-/- Mice)

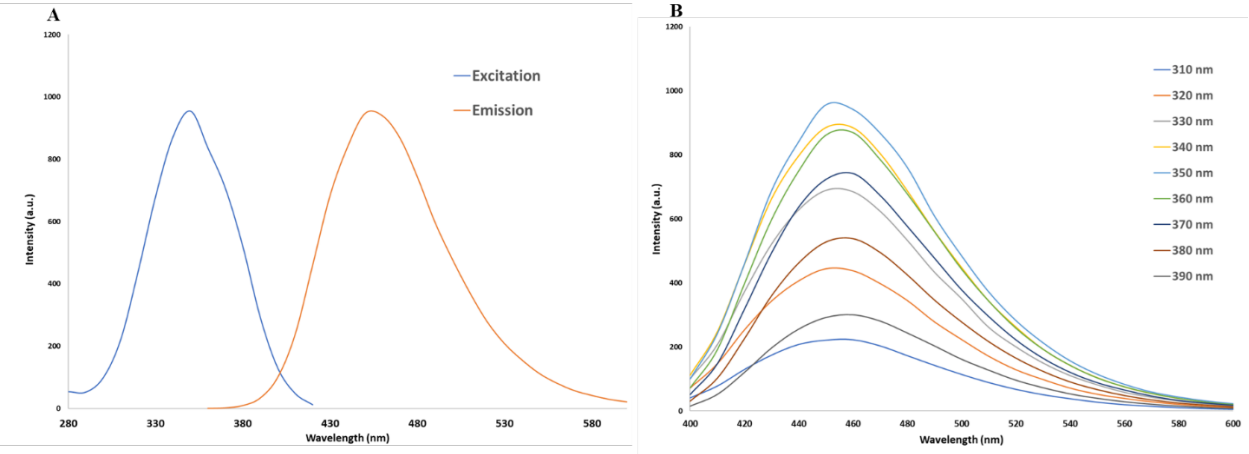
Thirty-six 6-week-old male APOE-/- mice were purchased from the Jackson Laboratory. These mice were randomly placed in cages and held for isolation for one week, with three mice per cage. Animals were fed an atherogenic diet (15% fat by weight, 0.25% cholesterol, Harlan Teklad) one week prior to the study beginning. The control cage was randomly picked, as well as the three experimental groups, which each consisted of nine subjects. For 16 weeks, all mice were dosed twice per week. Control mice were dosed with saline via intraperitoneal injection. The experimental mice were dosed with the same method as the control but were given carbon nanodots (0.5mg/kg BW). The mice were fed the atherogenic diet as mentioned previously, through the course of the study as well, with food intake and BW being measured at the beginning of the study and weekly. Once the 16-week dosing period was completed, the animals were humanely euthanized using isoflurane. A cardiac puncture and cervical dislocation were performed. The heart tissue was collected and stored in the -80°C freezer for further analysis.

CHAPTER III: RESULTS

Characterization of CNDs

To characterize the CNDs, the Cary Eclipse Fluorescence Spectrophotometer was used, which revealed the photoluminescent characteristics of CNDs. This was displayed through the excitation wavelength of around 345 nm and an emission peak of around 460nm (Fig. 1a). By understanding the specific excitation and emission spectra of CNDs, this information can be applied to detect these nanoparticles during uptake assays. CNDs were further studied through excitation with various wavelengths from 320-290 nm while showing consistent emission peaks at 460nm (Fig. 1b).

Figure 1. Characterization of CNDs.

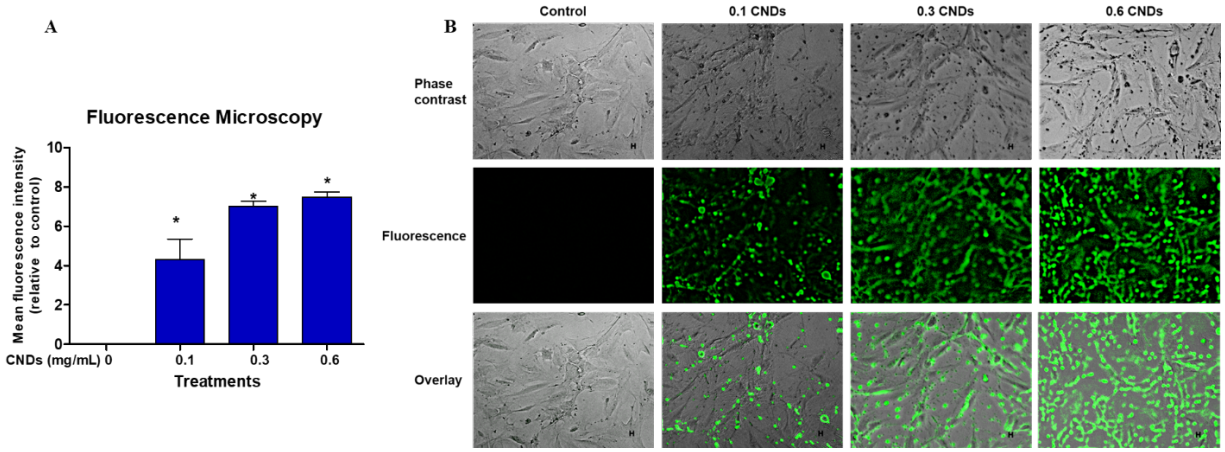


Characterization of CNDs. UV-Vis photoluminescence in a.u. measured by Cary® Eclipse™ Fluorescence Spectrophotometer. (a) Excitation ranged from 280-430nm with a peak at 345nm and Emission ranged from 360-600nm with a peak at 460nm. (b) CNDs display emission peak of ~460 nm with excitation wavelengths from 320nm-390nm.

CNDs Cellular Uptake

To understand the function and role of CNDs in cells, it is crucial to understand if endothelial cells have the ability to uptake nanoparticles to produce effects. To measure this, an uptake assay was performed by treating HAEC at 24-hour treatment with 0-0.6 mg/mL CNDs. A fluorescence microscope was used to image each unfixed well containing HAEC with various treatments. Fluorescence intensity was quantified using ImageJ (Fig 2a). As shown in Figure 2a, different concentrations of CNDs significantly increased the fluorescence intensity ($p<0.05$) compared to the control, indicating increased uptake of CNDs. The fluorescence uptake images include phase contrast images, fluorescence images and overlay images (Fig 2b).

Figure 2. CNDs Cellular Uptake Using Fluorescence Microscopy.

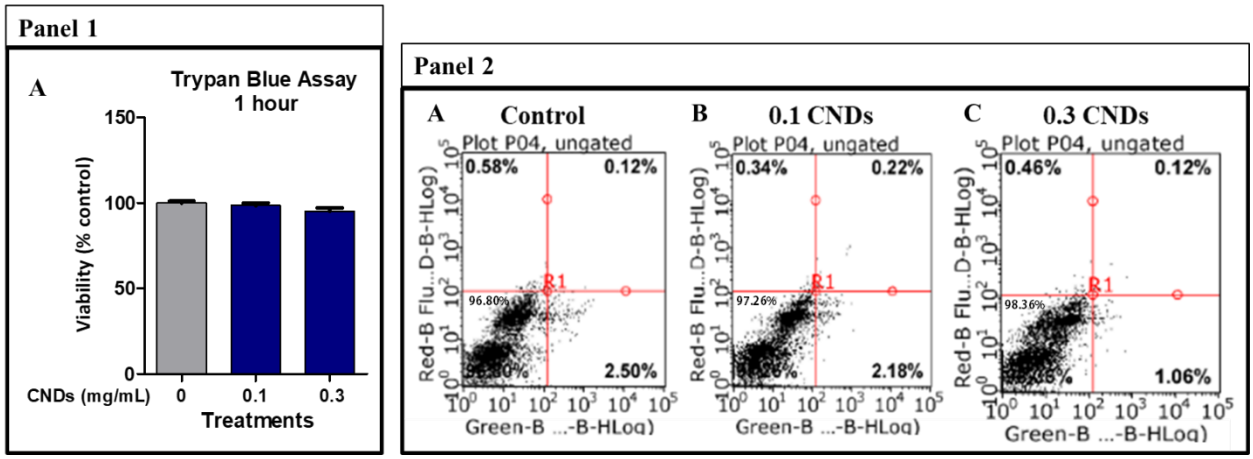


CNDs Cellular Uptake Using Fluorescence Microscopy. HAEC were treated with 0.1, 0.3, and 0.6 mg/mL CNDs for a duration 24 hours. Cells were cultured in 6 well plates and viewed with the fluorescence microscope. (a) Quantification of HAEC (n=3) fluorescence intensity using ImageJ Intensity. (b) Representative images show phase contrast, fluorescent and overlayed images of each concentration (n=3).

Cell Viability using Trypan Blue and Nexin (Flow Cytometry)

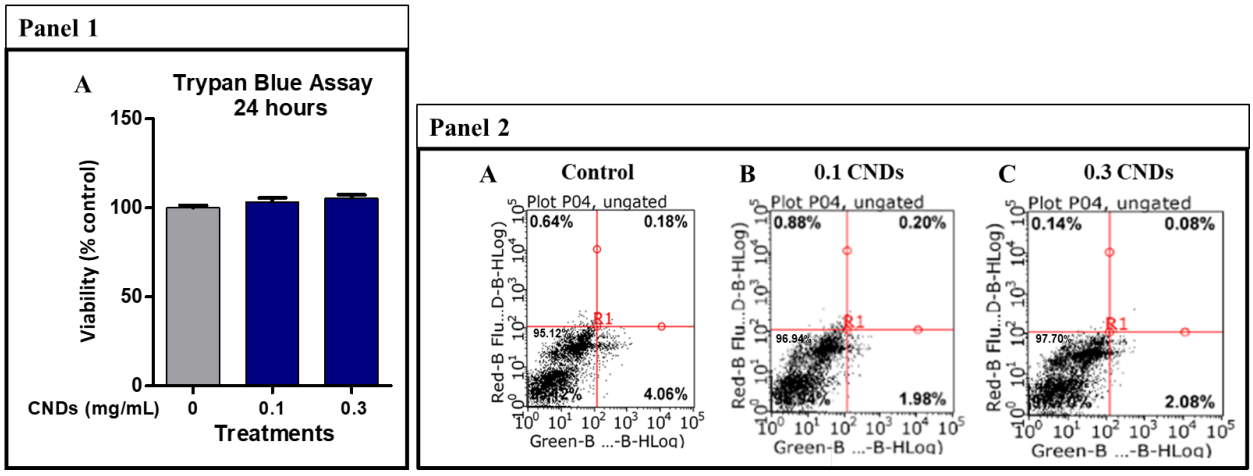
To further understand if CNDs can potentially cause toxicity to the cells, we measured cell viability using Trypan blue and flow cytometry. Toxicity to the cells, especially necrosis, is known to cause inflammation. Trypan Blue cell staining was first used to measure the cell viability. When the cell membrane has been compromised, the dye is able to enter the cell and bind to the proteins in HAEC, turning the cells color blue. Trypan blue is a dye that has the ability to permeate through cells only if there is damage to the cell membrane. For this assay, HAEC were treated with 0, 0.1 and 0.3 mg/mL of CNDs for 1 and 24 hours in 6-well Corning cell-culture treated plates. Cells were collected and spun down in a centrifuge. Our results displayed no significant change in cell viability to HAEC with any of the CNDs doses used for either time treatment, compared to the control ($p<0.05$) (Figs 3-4. Panel 1a). Nexin Flow Cytometry was further used to confirm the effects of CNDs on cell viability and type of cell deaths involved. To quantify the amount of apoptosis, FITC Annexin V was used. FITC Annexin V is used to be able to detect apoptosis through its high affinity for phosphatidylserine (PS) protein which translocate outside the cell when cell death occurs. FITC Annexin V can bind to the PS on the cell surface which then emits fluorescence which can be measured using flow cytometry. 7-AAD dye was used to read intensity of the dye to detect necrotic cells. 7-AAD dye cannot penetrate the cell membrane unless there is damage to the membrane. Once this occurs, the dye can enter and bind to double-stranded DNA and the fluorescence can be measured with flow cytometry. Flow cytometry is used to quantify the number of viable cells, cells in early or late apoptosis and necrotic cells as shown in Figs 3-4, Panel 2a, 2b, 2c. At the CNDs concentrations used, there were no significant toxic effects when compared to the control. This suggests that there are no toxic effects of CNDs at 0.1, 0.3 or 0.6 mg/mL.

Figure 3. Cell Viability Using Trypan Blue and Flow Cytometry: 1 Hour.



Cell Viability Using Trypan Blue and Flow Cytometry Assay for HAEC cells exposed to CNDs at 0.1 and 0.3 mg/mL CNDs for 1 hour. HAEC were treated with 0.1 and 0.3 mg/mL CNDs for a duration of 1 hour. FITC Annexin V and 7-AAD were used to measure fluorescent intensity to detect necrosis and apoptosis. HAEC were treated for 1 hour and then Trypan Blue was used to determine cell viability shown in Panel 1A. HAEC were treated as follows in panel 2:(a) control, (b) 0.1 mg/mL CNDs, and (c) 0.3 mg/mL CNDs. The percentage of viable, early apoptotic, late-apoptotic and necrotic cells were measured.

Figure 4. Cell Viability Using Trypan Blue and Flow Cytometry: 24 Hours.



Cell Viability Using Trypan Blue and Flow Cytometry Assay for HAEC cells exposed to CNDs at 0.1 and 0.3 mg/mL CNDs for 24 hours. HAEC were treated with 0.1 and 0.3 mg/mL CNDs for a duration of 24 hours. FITC Annexin V and 7-AAD were used to measure fluorescent intensity to detect necrosis and apoptosis. HAEC were treated for 24 hours and then Trypan Blue was used to determine cell viability shown in Panel 1A. HAEC were treated as follows in panel 2:(a) control, (b) 0.1 mg/mL CNDs, and (c) 0.3 mg/mL CNDs. The percentage of viable, early apoptotic, late-apoptotic and necrotic cells were measured.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) for Proinflammatory Genes

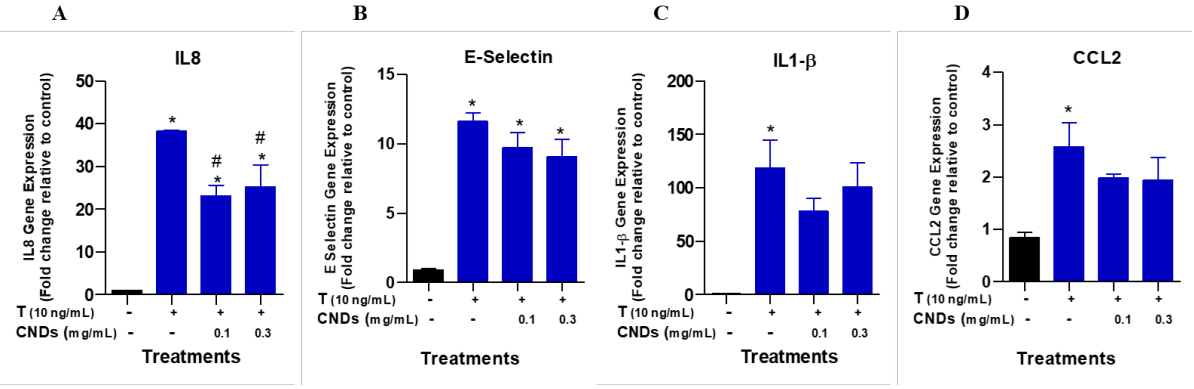
With TNF-α being a key regulator of atherosclerosis, it is critical to observe the effects TNF-α has on HAEC and how CNDs treatments can change gene expression. Proinflammatory cytokines are critical to study due to their promoted expression through different factors and pathways and their ability to amplify inflammatory effects. We wanted to investigate the effect of CNDs on TNF-α-induced proinflammatory biomarkers in HAEC endothelial cells. HAEC were treated with 0.1 or 0.3 mg/mL of CNDs with TNF-α at a concentration of 10 ng/mL for 1

hour. Once treatment was completed, cells went through RNA extraction, cDNA synthesis and qRT-PCR analysis.

IL-8 is an important biomarker with its role in neutrophils and immune cell recruitment to sites of infection. Gene expression of IL-8 showed a significant decrease ($p<0.05$) in cells co-treated with either 0.1 or 0.3 mg/mL of CNDs and 10 ng/mL of TNF-α for 1 hour compared to treatment with TNF-α alone (Fig 5a). E-Selectin is another critical biomarker that was measured due to its neutrophil and monocyte recruitment abilities. As shown in Fig. 5b, the proinflammatory gene expression of E-Selectin showed an approximately 12-fold statistically significant change ($p < 0.05$) between the control and TNF-α treatments. CNDs at 0.1 and 0.3 mg/mL were able to reduce TNF-α mediated expression of E-Selectin by a fold change decrease to 9.7 and 9, respectively.

It is shown that TNF-α treatment significantly increased gene expression of proinflammatory gene IL-1β and CCL2 (Fig. 5 c-d) as compared to the control. There is an overall trend seen that varying doses of CNDs can lead to a decrease in this TNF-α-induced expression of IL-1β and CCL2. When co-treated with TNF-α, a decrease in IL-1β expression was observed for both 0.1 and 0.3 mg/mL of CNDs, with a fold change from 118 for TNF-α treatment alone to 78.22 and 100.7, respectively. CCL2 was also analyzed for its critical role in the recruitment of macrophages during inflammation. A decrease in CCL2 expression was seen when cells were co-treated with TNF-α and 0.1 and 0.3 mg/mL of CNDs with a fold change from 2.58 for TNF-α treatment alone to 1.98 and 1.93.

Figure 5. Effects of CNDs on the Gene Expression of Proinflammatory Cytokines.



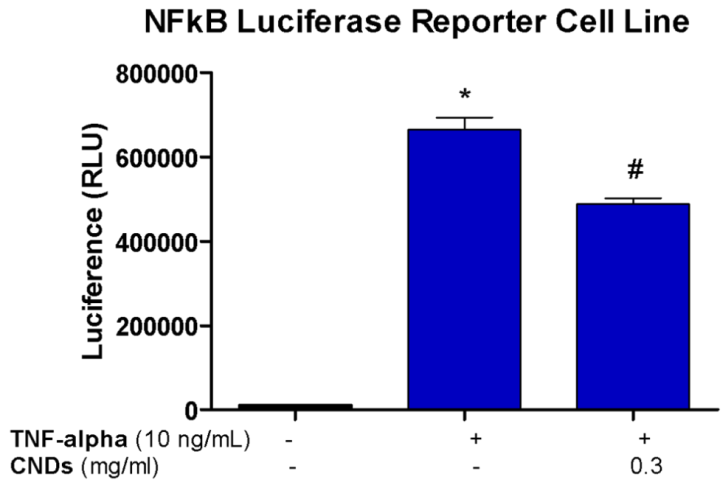
Effects of CNDs on the Gene Expression of Proinflammatory Cytokines. HAEC cells were treated with or without 10 ng/mL TNF-α and 0.1 or 0.3 mg/mL of CNDs in the medium for a duration of 1 hour. Following this treatment, RNA extraction, cDNA synthesis and qRT-PCR were performed to analyze the expression of (a) CCL2, (b) E-Selectin, (c) IL1-B, and (d) IL8. The results of gene expression analysis demonstrated a significant change in E-Selectin and IL8. (n=3, All results are represented as mean ± SEM. *, $p<0.05$ vs. control; #, $p<0.05$ vs. TNF-α alone)

NF-κB Luciferase Reporter Cell Line

The NF-κB pathway is a significant route through which there is a regulation of proinflammatory cytokines and adhesion molecules, which TNF-α takes. Signosis developed an NF-κB luciferase reporter stable cell line, which is an excellent model due to the transfecting NF-κB luciferase reporter vector. NF-κB reporter cells were treated with or without 10 ng/mL of TNF-α and 0.3 mg/mL of CNDs in a complete growth medium. Upon completion of treatment, the cells were washed with PBS and lysis buffer, and luciferase substrate was added to increase the luminescence of NF-κB if it was activated. NF-kB transcription factors are able to bind to their response elements, inducing transcription of the luciferase reporter gene, which can thus be measured in terms of fluorescence intensity. The plate was read in Bio-Tek Synergy plate reader,

and the luciferase assay was conducted to measure RLU of 0.3 mg/mL CNDs on TNF-α induced inflammation, and it was seen that TNF-α was able to increase the fluorescence intensity through increasing the NF-kB activity. When co-treated with 0.3 mg/mL of CNDs, there was a significant decrease demonstrated as compared to the TNF-α treatment alone ($p<0.05$) (Fig 6).

Figure 6: NF-κB Luciferase Reporter Cell Line.



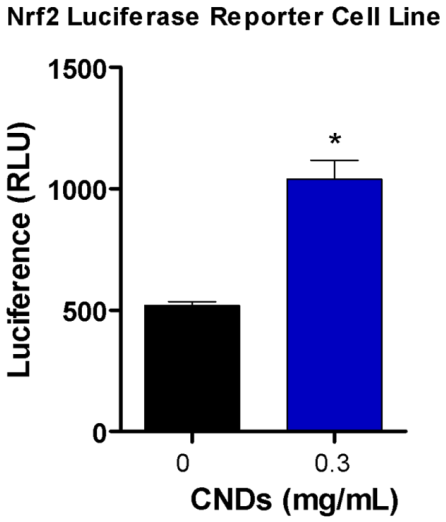
NF-κB Luciferase Reporter Cell Line: NF-κB cells were treated with or without 0.3 mg/mL of CNDs and 10ng/mL of TNF-α in a complete growth medium. Lysis buffer and luciferase substrate were added, and plate was read in Bio-Tek® Synergy™ plate reader. Luciferase assay was conducted to measure RLU of 0.3 mg/mL of CNDs on TNF-α-induced inflammation.

Nrf2 Luciferase Reporter Cell Line

The Nrf2 pathway is a cellular defense mechanism that plays an important role in protecting cells from oxidative stress and inflammation. When activated, Nrf2 regulates the expression of various antioxidant and anti-inflammatory genes, which help to reduce inflammation. Understanding the Nrf2 pathway can help Nrf2 luciferase reporter cell line was

purchased from Signosis, which was developed by co-transfecting the Nrf2/ARE luciferase reporter vector and hygromycin expression vector. Nrf2 reporter cells were treated with or without 0.3 mg/mL of CNDs in a complete growth medium. Lysis buffer and luciferase substrate were added, and plate was read in Bio-Tek® Synergy™ plate reader. Luciferase assay was conducted to measure RLU of 0.3 mg/mL of CNDs on TNF-α-induced inflammation. Upon completion of treatment, the cells were washed with PBS and lysis buffer and luciferase substrate were added to increase the luminescence of Nrf2 if it was activated. The plate was read in Bio-Tek Synergy plate reader and the luciferase assay was conducted to measure RLU of 0.3 mg/mL CNDs, which demonstrated a significant increase ($p<0.05$) (Fig 7).

Figure 7. Nrf2 Luciferase Reporter Cell Line.



Nrf2 Luciferase Reporter Cell Line. Nrf2 cells were treated with or without 0.3 mg/mL of CNDs in a complete growth medium. Lysis buffer and luciferase substrate were added, and plate was read in Bio-Tek® Synergy™ plate reader. Luciferase assay was conducted to measure RLU of 0.3 mg/mL of CNDs on TNF-α-induced inflammation.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) for Anti-inflammatory Genes

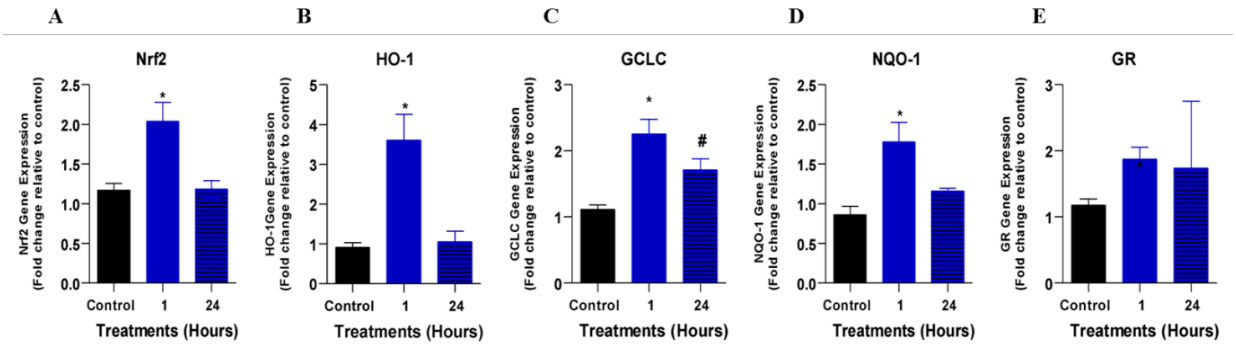
TNF-α has been found to cause an inflammatory immune response associated with releasing O2-· and OH· and anti-inflammatory genes are known for their roles in mediating inflammation. We were interested in determining whether the anti-inflammatory properties of CNDs are related to possible changes in the expression of anti-inflammatory genes, including Nuclear factor erythroid 2 (Nrf2), NAD(P)H: Quinone Oxidoreductase 1 (NQO1), Heme Oxygenase 1 (HO-1), Glutamate-Cysteine Ligase Catalytic Subunit (GCLC) and Glutathione reductase (GR). HAEC cells were treated with 0.3 mg/mL of CNDs for a duration of 1 hour. Following this treatment, RNA extraction, cDNA synthesis and qRT-PCR were performed to analyze the expression of HO-1, NQO-1, Nrf2, GCLC and GR.

Nrf2 is a transcription factor that is responsible for activating the expression of promoters at the antioxidant response element (ARE), which are responsible for maintaining the levels of reactive oxygen species (ROS) in cells. These promoters are activated when Nrf2 binds to them. When HAEC were treated with 0.3 mg/mL of CNDs, there was a significant increase ($p < 0.05$) seen in the expression of Nrf2 during the 1-hour treatments as compared to 24 hours (Fig 8a). Another important antioxidant enzyme is GR as it is involved in catalyzing the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH). A similar trend has been observed in the GR antioxidant gene as well, with an increase in gene expression with the 1-hour treatment (Fig 8e).

HO-1 is an important antioxidant that is involved in the protection of endothelial cells from apoptosis. Through previous studies, it has been shown that when there is an overexpression of HO-1 occurring, this can lead to a reduction in TNF-α induced gene

expression of E-Selectin, ICAM, and VCAM. The gene expression of HO-1 when HAEC were treated with 0.3 mg/mL of CNDs showed a significant increase ($p < 0.05$) (Fig 8b). GCLC is important as it is a rate-limiting enzyme of glutathione synthesis, which is an important antioxidant molecule that helps protect cells from oxidative stress. A significant increase ($p < 0.05$) was observed in GCLC gene expression when HAEC were treated with 0.3 mg/mL CNDs for 1 hour and 24-hour treatments (Fig 8c). NQO1 is an important enzyme that plays a critical role in protecting cells against oxidative stress and inflammation and plays a role in regulating the activity of transcription factors such as NRF2 and NF-κB. Results for gene expression of NQO-1 when HAEC were treated with 0.3 mg/mL of CNDs showed a significant increase ($p < 0.05$) in gene expression for 1-hour treatments (Fig 8d).

Figure 8. Effects of CNDs on Anti-Inflammatory Gene Expression.

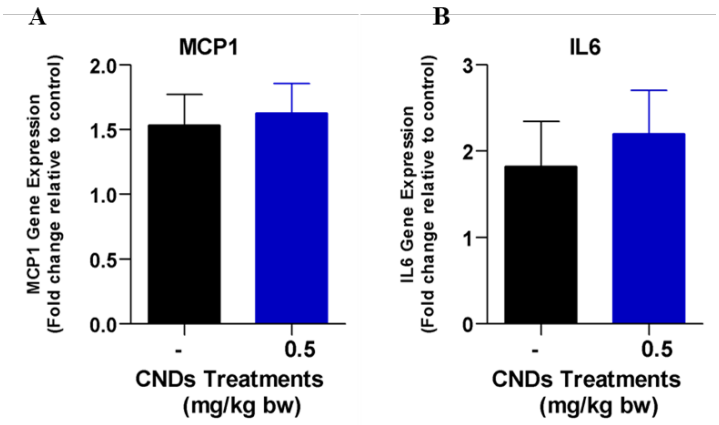


Effects of CNDs on Anti-Inflammatory Gene Expression. HAEC cells were treated with 0.3 mg/mL of CNDs for a duration of 1 or 24 hours. Following this treatment, RNA extraction, cDNA synthesis and qRT-PCR were performed to analyze the expression of (a) Nrf2, (b) HO-1, (c) GCLC, (d) NQO-1 and (e) GR. (n=3, All results are represented as mean ± SEM. *, $p<0.05$ vs. control)

Animal Project (APOE-/-)

To examine if the effects of CNDs in vitro have the same effect in vivo, apolipoprotein E (APOE) knockout mice will be used. APOE knockout mice are a commonly used mouse model for atherosclerosis research. This is due to APOE being critical for the clearance of cholesterol and lipoprotein particles from the blood. In humans, it has been seen that there is an increased susceptibility to atherosclerosis when there is a mutation in APOE. In APOE knockout mice, there is an increased level of plasma cholesterol and the development of atherosclerotic plaques. It is inferred that APOE knockout mice more closely represent human atherosclerosis due to the amount of cholesterol carried in LDL particles corresponding to human levels. Our in vivo qPCR results showed no significant change in the gene expression of MCP-1 or IL6 in the heart tissue samples treated with 0.5 mg/kg bw CNDs compared to control. MCP-1 and IL6 are chemokines in the recruitment of monocytes to sites of inflammation. This result indicates that 0.5 mg/kg bw of CNDs has no effect on heart tissue. Future studies are needed to study the effect of different doses of CNDs on the gene expression of more chemokine or adhesion molecules in the heart and other tissues such as the aorta (Fig.9).

Figure 9. Effects of CNDs in APOE-/- Mice on Proinflammatory Gene Expression in Heart Tissue.



Effects of CNDs in APOE-/- Mice on Proinflammatory Gene Expression in Heart Tissue. Mice were intraperitoneally injected with 0.5 mg/kg body weight of CNDs for a duration of 16 weeks. Mice were euthanized and organs were isolated. RNA extraction, cDNA synthesis and qRT-PCR were performed on heart tissue to analyze the expression of (a) MCP1, and (b) IL6. (n=6-9, All results are represented as mean ± SEM. *, $p<0.05$ vs. control)

CHAPTER IV: DISCUSSION

This study examined the effects of CNDs on TNF-α induced gene expression of proinflammatory and anti-inflammatory cytokines through different pathways in HAEC. It was observed that CNDs at a non-cytotoxic concentration significantly reduced TNF-α induced IL8 gene expression as compared to the TNF-α treatment alone. Through fluorescence microscopy, it was determined that HAEC were able to uptake CNDs in a dose-dependent manner. After examining the various pathways involved in inflammation, it was determined that CNDs increased Nrf2 transcriptional activity, which led to Nrf2-mediated upregulation of several antioxidant genes, including HO-1, GCLC, NQO-1, and GR. This led us to the conclusion that CNDs are able to upregulate cellular antioxidant defense mechanisms while suppressing endothelial cell inflammation. NF-kB Luciferase Reporter Cell line results showed that CNDs were able to reduce TNF-α-mediated increase in NF-kB activity. Through these results, it was suggested that CNDs can reduce inflammation through their role as a down-regulator of the NF-kB pathway and an up-regulator of the Nrf2 pathway.

Atherosclerosis is a chronic inflammatory disease triggered by endothelial cell dysfunction leading to inflammation and cellular dysfunction. Many previous studies demonstrated that proinflammatory mediator tumor necrosis factor TNF-α is a critical regulator of atherosclerosis, along with cell adhesion molecules and chemokines that are secreted by damaged endothelial cells. To study arterial disease, inflammation, and atherosclerosis, human aortic endothelial cells (HAEC) have been used because of their ability to be used for adhesion assays with several human monocytes. There are limited studies looking at the effects of CNDs on the cardiovascular system, and with endothelial cells being a critical component in the homeostatic balance, they are essential to study. The lining of the aorta is made up of endothelial

cells, and these cells are responsible for plate aggregation, response to neurotransmitters, inflammation, and various hormones [42]. They are in charge of the permeability, diameter, and blood flow rate in blood vessels. Disruption of HAEC can lead to endothelial cell dysfunction and atherosclerosis [43].

IL8 and IL-1β are two proinflammatory cytokines that play a critical role in recruiting and activating monocytes during inflammatory responses. It is known that IL-8 is highly expressed when endothelial dysfunction occurs in endothelial cells explaining the gene's role in mediating monocyte recruitment to inflammatory sites. Another very important cell adhesion molecule is E-Selectin, as it works to recruit white blood cells to inflammation sites and is upregulated in response to inflammatory signals. Our results showed that the exposure of HAEC to 0.1 and 0.3 mg/mL of CNDs decreased TNF-α induced relative gene expression of IL-8 and E-Selectin when compared to the TNF-α treatment alone. This suggests that CNDs have potential anti-inflammatory abilities that lead to a reduction of atherosclerosis by inhibiting proinflammatory cytokine and cell adhesion molecule expression. From previous studies conducted in our lab, similar results were seen when human microvascular endothelial cells (HMEC) were used, but this is the first study to look at the effects of CNDs on HAEC. It is unclear why the in vivo mouse trial of CNDs treatments had no effect on the gene expression of two key proinflammatory cytokines: MCP-1 and IL-6, in the heart tissue of APOE knockout mice. Future studies are needed to examine the effects of CNDs on aorta tissue, as it is extremely relevant to atherosclerosis. More cytokines, especially IL8, need to be studied further, as there has been a significant reduction through CNDs seen in HAEC.

The NF-κB pathway is a significant route for regulating proinflammatory cytokines and adhesion molecules. Extensive research has characterized the properties of NF-κB and

demonstrated that its activation plays a vital role in leukocyte adhesion to the endothelium. This is achieved through the transcriptional regulation of chemotactic cytokines and vascular adhesion molecules. The NF-κB transcription factor is initially located in the cytoplasm and remains inactive due to its inhibitor I-κB being bound. The p50/p65 heterodimer of NF-κB cannot enter the cell nucleus while bound to the inhibitor. However, through the phosphorylation and ubiquitination of the inhibitor, the NF-κB p50/p65 heterodimer is released and can translocate to the nucleus, where it binds to promoter regions. This phosphorylation and ubiquitination can be caused by TNF-α. This, in turn, stimulates the increased production of cytokines, chemokines, and adhesion molecules, signaling the immune system to respond. Through using an NF-kB luciferase reporter cell line, our results displayed TNF-α increasing NF-kB activity, whereas CNDs reduced the increased NF-kB activity. These results suggest that the ability of CNDs to reduce cytokine and adhesion molecules may be related to CNDs' role as a down-regulator of the NF-kB pathway. However, how CNDs can regulate the NF-kB pathway specifically remains unclear. Further studies need to be conducted to determine the specific location in the pathway that is being affected to cause these downstream effects. The most common dimer in the NF-kB signaling is the p65 homodimer. The translocation of p65 homodimer can be viewed using confocal immunofluorescence through treating cells with antibodies which can thus bind to the proteins that are produced in the pathway. This will allow the pathway to be imaged to see if TNF-α is able to trigger the pathway and if CNDs can inhibit the homodimer.

In addition to NF-kB, Nrf2 is another transcription factor that plays a critical role in regulating cellular responses to stress, inflammation, and oxidative stress. There has been documentation suggesting that there are crosstalk capabilities between the NF-kB pathway and Nrf2 signaling, which can negatively affect the activation of Nrf2 by causing competition for the

transcriptional coactivator (CREB-binding protein)-p300 complex. Nrf2 is present in the cytoplasm of cells under normal circumstances in vivo, and the capacity to maintain low levels of free Nrf2 is related to the Keap-1 repressor protein [13]. When keap-1 binds to Nrf2 in the cytoplasm, it causes it to translocate to the nucleus, where it interacts with antioxidant response elements, resulting in the production of antioxidant proteins [16]. The purpose of the Nrf2 system is to maintain cellular homeostasis and it can be considered an anti-stress mechanism [16]. A Nrf2 luciferase reporter cell line was used to explore whether Nrf2 is potentially activated, through the cell lines designed to fluoresce upon the addition of coelenterazine as a substrate when Nrf2 is activated. Our results indicated that treatments with CNDs significantly increased luminescence intensity, suggesting enhanced Nrf2 signaling. In order to assess if CNDs exposure can also trigger Nrf2-mediated antioxidant responses, Nrf2 target genes were measured. Consistent with the Nrf2 luciferase reporter assay results, CNDs significantly induced many Nrf2 target genes, including HO-1, NQO-1 and GCLC, while GR was unaffected. Upregulation of the HO-1 gene in the Nrf2 pathway has been shown to reduce the production of proinflammatory cytokines triggered by the NF-kB pathway. HO-1 is very important in inflammation as it has anti-inflammatory and antioxidant properties, leading to its ability to decrease apoptosis and cellular distress. HO-1 is also a critical component in the Nrf2 mediated NF-κB inhibition of the pathway by being a mediator for the crosstalk between the two pathways. The increase in Nrf2 activity, as viewed in the Nrf2 reporter cells, and significant induction of Nrf2 target genes indicate that Nrf2 activation plays a role in explaining the anti-inflammatory abilities of CNDs against TNF-α induced endothelial inflammation, as shown in Figure 5. Additional research is required to investigate if Nrf2 activation through CNDs can

impede NF-kB signaling by preventing the degradation of IkBa, leading to the inhibition of NF-kB-mediated gene expression and subsequently reducing inflammation.

In summary, our study provides for the first time in vitro model that CNDs reduce TNF-α induced expression of inflammatory genes, including IL8, E-Selectin, and CCL2. CNDs did not exhibit any cytotoxic capabilities in our results, with concentrations up to 0.6 mg/mL of CNDs used. Through our fluorescence microscopy results, we were able to determine that HAEC was able to uptake CNDs. The NF-kB luciferase reporter cell assay results displayed that CNDs could inhibit TNF-α-induced NF-kB activity. Our results further suggested that CNDs can activate Nrf2 transcription along with increasing Nrf2-mediated upregulation of various antioxidant genes, including HO-1, GCLC, NQO-1, and GR. The findings of this study indicate that the anti-inflammatory effect of CNDs may be linked to the downregulation of the NF-κB pathway and the up-regulation of Nrf2 signaling. Importantly, this is the first investigation of CNDs' impact on inflammation in human aorta endothelial cells.

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APPENDIX A: TABLES

Table A1. Target Human Gene Sequences.

Target Human Gene	*Forward Primer*	*Reverse Primer*
GAPDH	5'-AGAACGGGAAGCTTGTCATC -3'	5'- GGAGGCATTGCTGATGATCT -3'
IL8	5'- CTCTGTGTGAAGGTGCAG TT -3'	5'- AAACTTCTCCACAACCCTCTG -3'
IL1-β	5'- CCAGCTATGAACTCCTTCTC -3'	5'- GCTTGTTCCTCACATCTCTC -3'
CCL2	5'- GCTCAGCCAGATGCAATCAA -3'	5'- GGTTGTGGAGTGAGTGGTCAAG -3'
E-Selectin	5'- AGCTACCCATGGAACACGAC -3'	5'- ACGCAAGTTCTCCAGCTGTT -3'
Nrf2	5'- GCGACGGAAAGAGTATGAGC -3'	5'- GTTGGCAGATCCACTGGTTT -3'
HO-1	5'- TCCGATGGGTCCTTACACTC -3'	5'- TAAGGAAGCCAGCCAAGAGA -3'
NQO-1	5'-TTACTATGGGATGGGGTCCA-3'	5'- TCTCCCATTTTTCAGGCAAC-3'
GCLC	5'-ACCATCATCAATGGGAAGGA–3'	5'- GCGATAAACTCCCTCATCCA-3'
GR	5'- CAGTGGGACTCACGGAAGAT -3'	5'- AAACCCTGCAGCATTTCATC -3'

Table A2. Target Animal Gene Sequences.

Target Animal Gene	*Forward Primer*	*Reverse Primer*
B- Actin	5'-AGCCATGTACGTAGCCATCC-'3	5'- CTCTCAGCTGTGGTGGTGAA-'3
MCP-1	5'-TTCCTCCACCACCATGCAG-'3	5'-CCAGCCGGCAACTGTGA-3
IL6	5'-CTGCAAGAGACTTCCATCCAGTT-3	5'AGGGAAGGCCGTGGTTGT-3