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Investigating the toxicology of intramuscular injected CNT-AB in mice followed by microwave hyperthermia.

Conner Clark

Investigating the toxicology of intramuscular injected CNT-AB in mice followed by microwave hyperthermia.

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in *The Department of Chemistry and Biochemistry.*

By
Conner Clark
Under the mentorship of *Dr. Eric Gato*

ABSTRACT

The advent of carbon nanotubes (CNTs) has led to a wide range of research in various fields including cancer therapy for targeting specific localized and site-specific treatment. Carbon nanotubes bound to tumor specific antibodies (Ab) offers specific treatment for cancer cells without affecting surrounding tissue. This treatment makes use of infrared absorptive properties of nanotubes to incinerate both the nanotube and its associated tumor *in vivo*. We seek to affirm the initial results of CNT in cancer therapy by investigating the toxicological effect in mice injected with CNT-Ab followed by microwave hypothermia. After 1-week post-injection, mice were sacrificed followed by the collection of blood serum, liver, kidney and other tissues for further analysis. Albumin, total protein, aspartate transferase (AST), and creatinine levels were assessed in the blood serum. Total protein concentration across the treatment groups was varied. There were no significant changes in albumin levels as compared to the control group. Group YE (.125 mg/ml anti-PSMA-MWCNT + Microwave) was found to have consistently high blood serum analyte levels indicative of impaired liver and kidney functioning. No other treatment groups seemed to have shown any evidence of impaired kidney function; however, groups YB (Microwave only), YF [.5 mg/ml anti-PSMA-MWCNT (No Microwave)], and YG (.5 mg/ml plain MWCNT + Microwave) seemed to show indications of impaired liver function. Analysis of gene expression revealed a significant impact on the NF-$_K$B inflammatory response pathway. NF-$_K$B gene was upregulated relative to controls in all treatment groups. These results seem to suggest marginal toxicity from the injection of ab-CNT followed by hyperthermia in mice subjects.

Thesis Mentor:_____

Dr. W. *Eric Gato*

Honors Director:_____

Dr. Steven Engel

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Introduction

Cancer is a cluster of diseases that involves abnormal and uncontrollable cell growth which has the potential to spread to other parts of the body. It is a genetic disease caused by the alteration of genes that control cell growth and division. With more than 100 types of cancer, it is the second highest leading cause of death in the United States [1]. The complexities of cancer make the disease extremely difficult to treat once it has invaded more than one tissue type. Cancer treatments such as chemotherapy, radiation therapy, and immunotherapy have serious side effects and the potential for biological resistance. Existing efforts that target single cellular abnormalities have met many obstacles yet have been proven to be effective [2].

Prostate cancer is one of the most lethal malignancies to men, second to lung cancer. Roughly 1 in 9 men will be diagnosed with prostate cancer and 1 out of 41 will die from it. Options for treatment are commonly surgical resection, while the remaining alternatives such as chemotherapy and radiation therapy have large potential for healthy cell death and undesired side effects [3]. Other established targeting therapies can have great shortcomings and low success rates.

Carbon nanotubes (CNTs) are nanostructured materials that are smaller than a single nanometer at one characteristic structural length [4]. Carbon nanotubes conjugated to antibodies (CNT-Abs) offers for specific binding to cancer cells without association to other normal cell types. This treatment makes use of the infrared absorptive properties of nanotubes to incinerate both the nanotube and its associated tumor in vivo thus diminishing or eliminating associated side effects of current cancer treatments that

damage surrounding tissues. The function of the CNT-Abs at the cellular level provides specialized targeting of desired cell types.

Previous studies have shown that CNTs have the potential to trigger inflammation, cytotoxicity and oxidative stress; however, there is not yet a full understanding of the toxicity of CNTs [5]. Further studies have demonstrated that the degree of toxicity is dependent on the surface chemistry of the CNTs. Functionalized carbon-based nanomaterials opposed to pristine carbon-based nanomaterials are expected to present a greater dispersibility in an aqueous medium, which could increase the cell response. CNTs associated with any metal have been found to have a higher chance for negative reactions [6]. The direct interaction between carbon-based nanomaterials and the cell can enhance oxidative stress within the cell due to CNTs ability to stimulate the generation of reactive oxygen species (ROS), which can damage lipids, carbohydrates, proteins, and DNA. This oxidative stress has been implicated in the induction of inflammation in many studies examining CNTs both in vivo and in vitro [7]. Effectiveness of CNT treatment has also been found to be dependent on the tissue type. For instance, CNTs' introduced to lung tissue had a significant negative impact on the elderly with respiratory problems [8].

This research seeks to affirm preliminary data about the effectiveness of CNT-Abs for targeted cancer treatment and to explore potential systemic and localized impact of CNT-Ab treatment followed by microwave hyperthermia. The importance of this study could provide new treatment options for a variety of cancers that would offer specific and localized cancer treatment without the invasiveness of surgery or the risk of tissue damage as in chemotherapy and radiation.

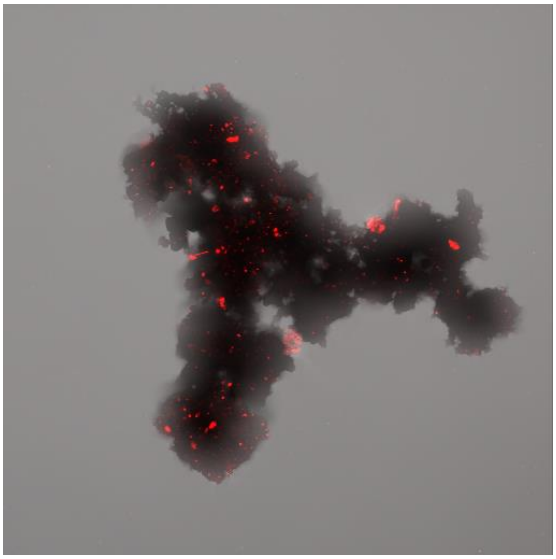

Figure 1: Functionalized multiwalled carbon nanotube used. Red color indicates the associated prostate cancer specific antibodies.

Materials and Methods

Experimental Design

Approximately 4-5 week old male mice were assigned to 8 different treatment groups, each group containing 6 mice. As specified in table 1, treatment groups vary in CNT concentration, microwave exposure, and presence of prostate cancer specific antibodies (Prostate membrane specific antigen-PSMA). The mice were injected with multiwalled carbon nanotubes (MWCNT) conjugated with cancer specific antibodies (CNT-Abs/ PSMA-MWCNT) subcutaneously into both right and left flank and microwaved at 150 watts for 5 seconds. After 1-week post-injection, blood samples were collected. Further, tissues such as liver, kidney, and brain tissue were obtained for future examination.

Mice were housed at the Georgia Southern University Animal Facility (1176A Biological Sciences Fieldhouse). The animals were handled according to the principles

outlined in the ILAR's (Institute or Laboratory Animal Research) Guide for Care and Use of Laboratory Animals. Study protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC Protocol Approval # I18024).

Table 1: Treatments assigned to each group of mice (Y group, one week post-injection).

Sample	Treatment
A	No Treatment
B	Microwave only
C	.125 mg/ml anti-PSMA-MWCNT (No Microwave)
D	.125 mg/ml plain MWCNT + Microwave
E	.125 mg/ml anti-PSMA-MWCNT + Microwave
F	.5 mg/ml anti-PSMA-MWCNT (No Microwave)
G	.5 mg/ml plain MWCNT + Microwave
H	.5 mg/ml anti-PSMA-MWCNT + Microwave

Serum samples were assayed to quantify systemic and localized impact of treatment. Further research onto the specific tissue types was conducted to analyze gene expression and the influence of treatment on immune and inflammatory responses.

Serum Creatinine Assay

The reagent was prepared to quantify creatinine using a 48 well plate. Reaction buffer was made up of a mixture of 1ml creatinine sodium borate, 3ml creatinine surfactant, and 2ml creatinine NaOH. A standard was then prepared using a series of dilutions of the creatinine standard and HPLC-grade water. Samples ranging from A through H (Table 1) were analyzed using guidelines provided by the assay manufacturer [Cayman Chemical, Creatinine (serum) Colorimetric Assay, Ann Arbor Mi; Cat# 700460]. Approximately 15 μl of each sample was added to the respective wells along with 100 μl of creatinine reaction buffer and 100μl of creatinine color reagent. The

absorbance of the solutions was read at 495nm after 1 minute and then 7 minutes while being incubated at room temperature. The creatinine concentrations were then determined via the standard curve.

Serum Albumin Assay

The level of albumin in serum was measured using a reagent kit provided by Pointe Scientific, Inc., (Canton, MI, Cat#: A7502-1L). Using guidelines provided by the kit manufacturer, albumin reagent was mixed with serum samples in their respective test tubes followed by incubation at room temperature. The UV spectrophotometer was blanked and then read and recorded at 630nm. Albumin concentrations were calculated using a standard curve generated by an albumin standard.

Serum Liquid AST (Aspartate Aminotransferase) Assay

Level of AST activity was also quantified in serum samples (Pointe Scientific, INC., Canton, MI, Cat#: A7561-450). Working reagents were pre-warmed followed by the addition of the respective serum samples using assay protocol provided by the kit manufacturer. Then the absorbance was read and recorded in a UV spectrophotometer at 340nm. Each sample was then returned to the incubator and absorbance was read again every minute for the next 2 minutes. The average difference in absorbance per minute was calculated and multiplied by the factor 1768 to yield IU/L. Samples were run at least thrice in duplicates.

Tissue RNA Extraction

Total RNA was extracted from liver, kidney and brain tissues using Qiagen's RNeasy mini RNA extraction kit. Approximately 20 to 30 milligrams of liver were weighed for samples A-H. Tissues were placed and homogenized in QIAzol Lysis reagent. Addition of gDNA eliminator solution and chloroform were performed followed by centrifugation at low temperature. Next, the RNA supernatant was removed, passed through an RNeasy spin column, and then the column was cleaned using RWT and RPE buffer. Finally, RNA samples were eluted from the spin columns using RNase free water. The quality and concentration of the total RNA were examined with a Nanodrop (Thermo Scientific Nanodrop, 2000/2000c Spectrophotometer, Cat#:ND-2000c) nucleic acid spectrophotometer. RNA gel electrophoresis was also performed to evaluate the quality of the RNA.

cDNA synthesis

Complementary DNA (cDNA) was synthesized from total RNA samples using the Bio-Rad's iScript Reverse Transcription Supermix for RT-qPCR (Hercules, CA, USA) following manufacturer guidelines.

Gene Expression

The expression of various genes related to inflammatory response in the treatment groups YA-YH was analyzed. Genes such as IL1B, IL6, PTSG2, TNF, and NFKB were analyzed using β-actin as a control gene. The reaction mixture comprised of forward and reverse primers specific to the gene of interest (table 2), SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), nuclease free water and cDNA. Reaction mixture was

run on a Bio-Rad CFX96 Rt-PCR system to quantitatively measure the expression of mRNA transcripts.

Table 2: Description of primers used for each gene studied.

Gene	Forward Primer	Reverse Primer
PTSG2	5'-GCGGGAACACAACAGAGTAT-3'	5'-GGACAGCCCTTCACGTTATT-3'
TNF-α	5'-ACAAGGCTGCCCCGACTAT-3'	5'-CTCCTGGTATGAAGTGGCAAATC-3'
IL-1β	5'-TACCTATGTCTTGCCCGTGGAG-3'	5'-ATCATCCCACGAGTCACAGAGG-3'
NF-kB	5'-AGCAGGATGCTGAGGATTCTG-3'	5'-GGCAACTCTGTCCTGCACCTA-3'
IL-6	5'-GGAGTTTGTGAAGAACAACT-3'	5'-CTAGGGTTTCAGTATTGCTC-3'

Data Analysis

The statistical analysis of data was performed using analysis of variance (ANOVA). Albumin concentration, creatinine concentration, total protein concentration, and AST activity were evaluated for significant changes comparing the treated groups to the control group. Significant differences between the treatment groups were denoted as either $*p < 0.05$ or $**p < 0.01$ and data were presented as mean ± standard error.

Results

Quantification of Albumin, AST and Creatinine and Total Protein Levels in Serum

An evaluation of the serum albumin concentration of the control and treatment groups were performed, which allowed a comparison of the differing treatment groups

and their systemic effects. Figure 1 illustrates the mean serum creatinine in mg/dl. Group YA (no treatment) is the control group. Compared to the control group, no significant change was noted in the treated groups.

Shown in Figure 2 is the serum creatinine concentrations of all experimental groups. Creatinine concentrations of groups YB (Microwave only), YD (.125 mg/ml plain MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT + No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave) were significantly reduced when compared to the control mice. Compared to the control groups, YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave) & YE (.125 mg/ml anti-PSMA-MWCNT + Microwave) treatment groups did not show any significant differences.

Evaluation of AST was performed across treatment and control groups. Figure 3 illustrates AST activity levels in mice for groups YA (No Treatment),YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), YD (.125 mg/ml plain MWCNT + Microwave), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave) for serum AST. Compared to YA (No Treatment), groups YB (Microwave only), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave) and YG (.5 mg/ml plain MWCNT + Microwave) showed significantly increased AST activity in blood serum. On the contrary, YD (.125 mg/ml plain MWCNT + Microwave) and YH(.5 mg/ml anti-PSMA-MWCNT + Microwave) mice appeared to show

significantly reduced levels of serum AST activity. No change was observed in the YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave) treated mice.

Total protein concentration is reported in Figure 4. Serum total protein concentration values mirror that of the albumin concentration. Although the treated groups were either slightly lower or higher, these values were within the margin of error. None of the treatment groups when compared with the control mice showed any significantly elevated or diminished total protein levels.

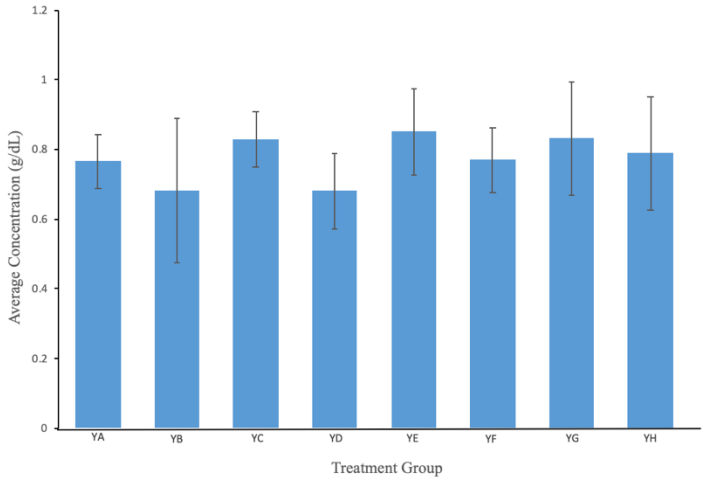

Figure 1: Mean Serum Albumin of treated mice. Treatment groups included: YA (No Treatment),YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), YD (.125 mg/ml plain MWCNT + Microwave), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

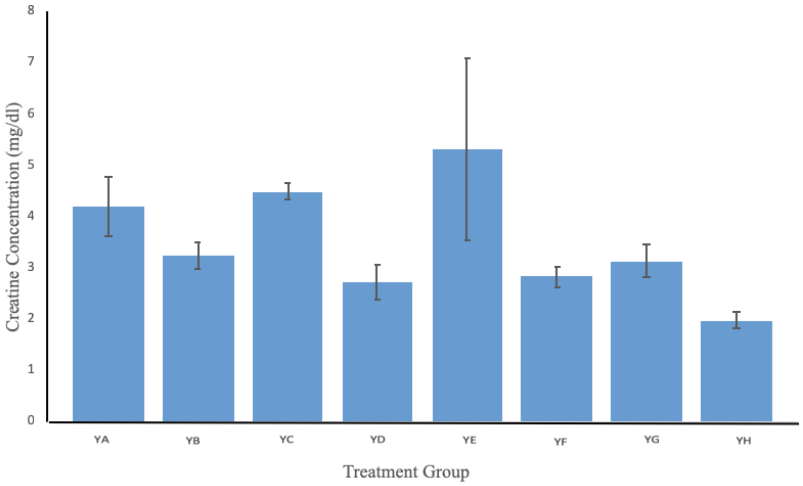

Figure 2: Mean Serum Creatinine in treated mice. Lower levels of creatinine were found in YB (Microwave only), YD (.125 mg/ml plain MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave), and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave) groups. Creatinine levels in YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave) and YE (.125 mg/ml anti-PSMA-MWCNT + Microwave) groups were not altered significantly.

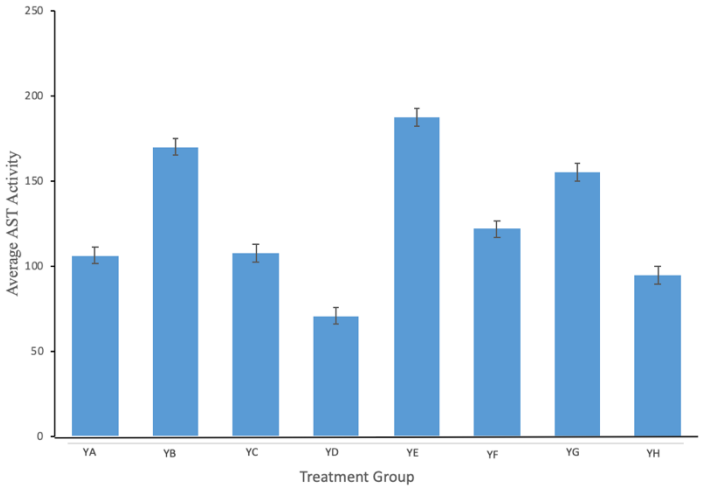

Figure 3: Mean serum AST activity (IU/L) in treated mice. Treatment groups included; YA (No Treatment),YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT + No

Microwave), YD (.125 mg/ml plain MWCNT + Microwave), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

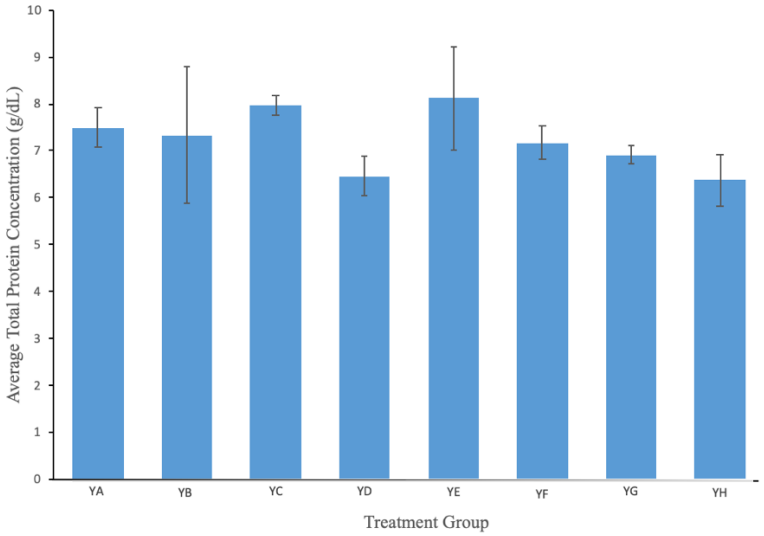

Figure 4: Mean Serum Total Protein of treated mice. Levels of total protein in blood serum were relatively constant for all groups. Treatment groups included; YA (No Treatment),YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), YD (.125 mg/ml plain MWCNT + Microwave), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Expression of inflammatory genes in the Brain, Liver and Kidney Tissues

Brain, hepatic, and renal relative expression of select inflammatory genes, IL1B, IL6, NF-$_K$B, PTSG2, and TNF, were measured in each experimental group. A brief description of each of these genes is provided in Table 3.

Gene expression of select inflammatory genes in the liver tissues of treated mice are displayed in Figure 5. Genes IL1B and PTSG2 were not expressed for any of the treatment groups. All treatment groups had a downregulation in expression for IL6 and TNF compared to the control. Groups YD (.125 mg/ml plain MWCNT + Microwave) and YE (.125 mg/ml anti-PSMA-MWCNT + Microwave) had an upregulation for NFKB, while YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT No Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), and YG (.5 mg/ml plain MWCNT + Microwave) were all downregulated when compared to the control.

Presented in Figure 6 is the gene expression in brain tissue across treatment groups. Across treatment groups, gene IL1B was not expressed and genes IL6, PTSG2, and TNF had a downregulation compared to the control. For the gene NFKB all treatment groups were upregulated compared to the control group in brain tissue.

The expression of select inflammatory genes in kidney tissue is shown in Figure 7 for all treatment groups. IL1B was not expressed for the treatment or control and gene TNF was down regulated across all treatment groups. Genes IL6 and PTSG2 were down regulated across treatment groups with exceptions to group YB (Microwave only) which was slightly upregulated. For the gene NFKB all treatment groups were upregulated compared to the control group.

Results portray the effect of treatment on important immune responses and whether or not a response is induced. Figures 5, 6, and 7 illustrate relative expression in

liver, brain, and kidney tissues respectively. IL1B was not expressed in any tissues across treatment groups. Genes Il6, PTSG2, and TNF were all under expressed across treatment groups and tissue types. Gene NF-$_K$B is of greatest interest due to the significant overexpression that most treatment groups portrayed across tissue types.

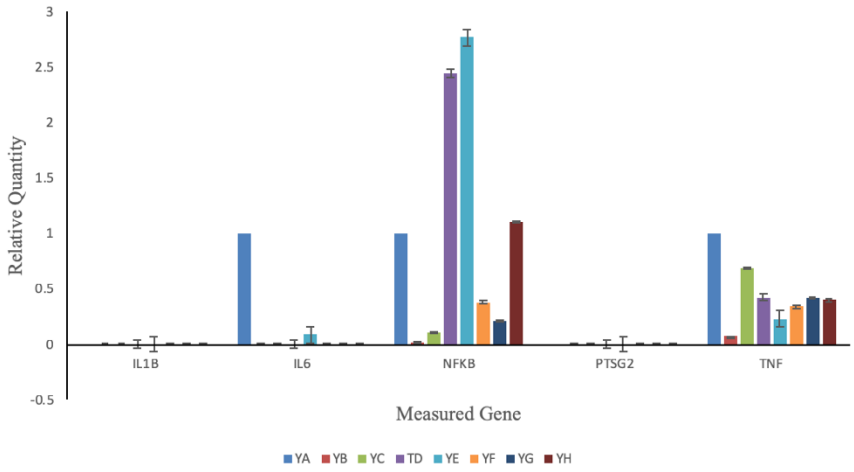

Figure 5: Relative gene expression in liver of treated mice. Treatment groups included; YA (No Treatment),YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), YD (.125 mg/ml plain MWCNT + Microwave), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

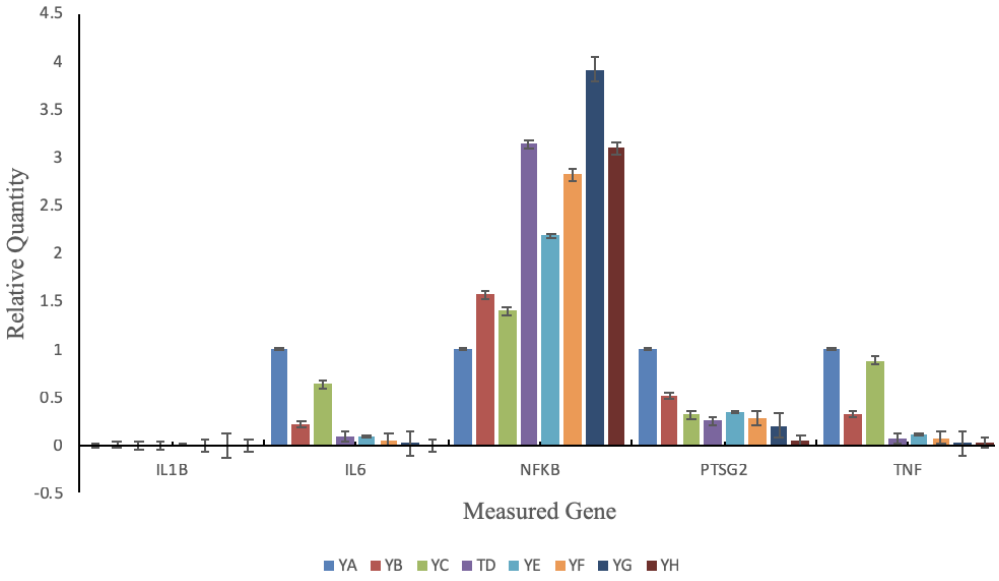

Figure 6: Relative gene expression in brain of treated mice. Treatment groups included; YA (No Treatment),YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), YD (.125 mg/ml plain MWCNT + Microwave), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

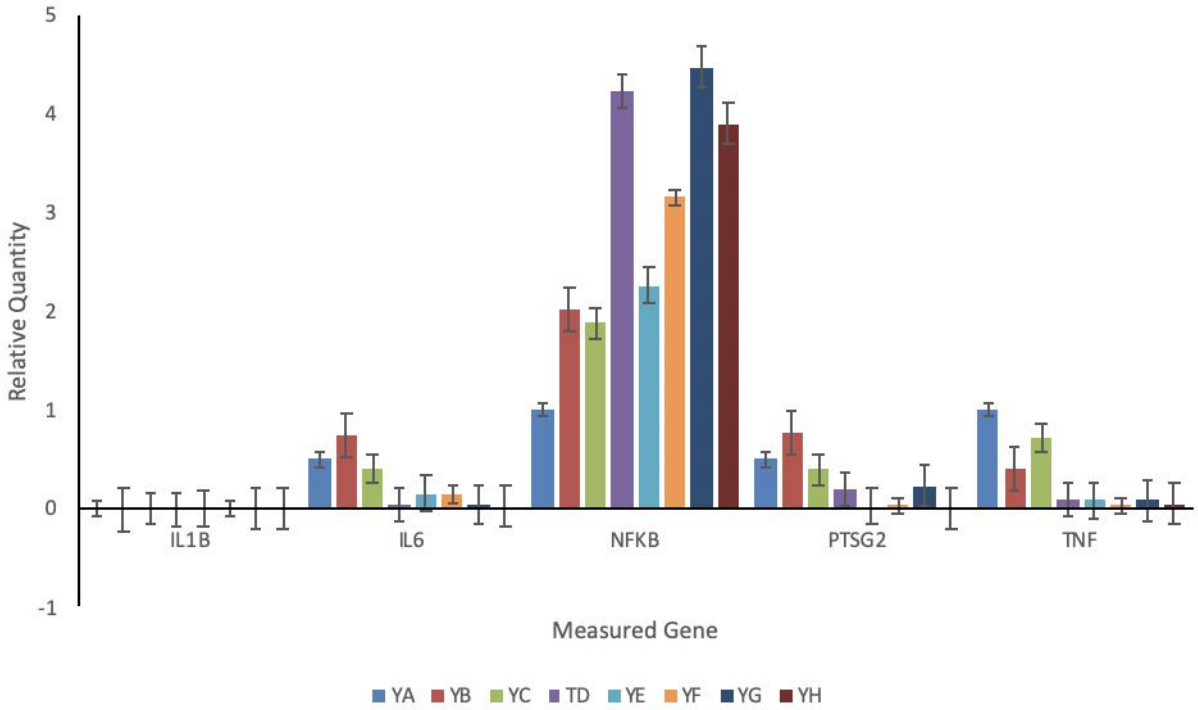

Figure 7: Relative gene expression in kidney of treated mice. Treatment groups included; YA (No Treatment),YB (Microwave only), YC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), YD (.125 mg/ml plain MWCNT + Microwave), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), YG (.5 mg/ml plain MWCNT + Microwave) and YH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Table 3: Description of genes studied including role, regulation, and signaling.

Gene	Description
IL1B	A member of the interleukin 1 cytokine family. The cytokine is produced by activated macrophages which is proteolytically processed to its active form by caspase. This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [9].

IL6	This gene encodes a cytokine that functions in inflammation and the maturation of B cells. The protein is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor, alpha [10].
NF_KB	NF_KB is a transcription regulator that is activated by various intra- and extra-cellular stimuli such as cytokines, oxidant-free radicals, and bacterial or viral products. Activated NFKB stimulates the expression of genes involved in a wide variety of biological functions. Inappropriate activation of NFKB has been associated with a number of inflammatory diseases [11].
PTGS2	This gene encodes the inducible isozyme PTGS2. It is regulated by specific stimulatory events and is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis [12].
TNF	This gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, and apoptosis. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer [13].

Discussion

Serum blood tests were conducted to study levels of creatinine, albumin, aspartate amino transferase (AST) and total protein within mice exposed to CNT conjugated antibodies. These assays provide insight into systemic impacts of the treatment including hepatotoxicity and nephrotoxicity, while investigation of gene expression provides insight into specific and localized effects on the kidney, brain, and liver tissues.

Creatinine is a waste product remaining from the metabolism of creatine phosphate in muscle. Creatinine assays reveal important information about kidney function and filtration rate as well. High levels of serum creatinine can thus indicate improper kidney function, decreased glomerular filtration rate and can also be indicative of intense exercise, dehydration, and high protein diets [14]. Mean serum creatinine levels of the treatment groups were significantly lower in comparison to the control mice with the exception of treatment group YE (.125 mg/ml anti-PSMA-MWCNT + Microwave) which was observed to have a much larger creatinine concentration. This indicates potential kidney impairment for the YE treatment group. Creatinine is formed largely in the muscles by the removal of water from creatine phosphate. While high levels of creatinine are not life threatening, it may be indicative of other underlying health issues such as chronic kidney disease. A study by Yadav and colleagues (2014) showed that serum creatinine levels are directly related to the severity of the disease and thus a great biomarker for kidney impairment [15].

Albumin is a globular blood transport protein that is the most abundant protein found in blood. Albumin binds substances including water, calcium, sodium, potassium, fatty acids, hormones and others. In addition to being a nutrient carrier, it also serves to regulate the oncotic pressure of blood. Low levels of serum albumin can indicate liver damage, kidney disease, malabsorption, malnutrition, and malignancies while high levels of albumin typically indicate dehydration [16]. There were no notable deviations in albumin levels across treatment groups compared to the controls.

Aspartate aminotransferase (AST) is an important enzyme in amino acid metabolism that is found predominantly in the liver, but also in smaller amounts in heart,

brain, muscle, and kidney tissues. AST levels serve as indicators of liver function and inflammation. Healthy individuals have naturally low AST levels, however when tissue that contains AST is damaged it is released into the blood leading to increased levels [17]. An increase in AST activity was found in groups YB (Microwave only), YE (.125 mg/ml anti-PSMA-MWCNT + Microwave), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), and YG (.5 mg/ml plain MWCNT + Microwave) for serum AST. These results suggest that CNT-Ab exposure might be indicative of potential damage to liver function within these treatment groups. A study by Hennes and colleagues on pediatric patients with blunt abdominal trauma were able to identify hepatic injury using AST as a biomarker with 100% sensitivity and 93% specificity. They found that AST levels had a significant correlation with positive results on CT scans used to diagnose patients. Elevated AST levels do not have a direct impact on any system but are a reliable biomarker for liver damage [18].

Proteins are important building blocks for organs, muscles, hormones, and enzymes and are therefore essential to the overall health of an organism. Fluctuations in the number of proteins in blood serum can result in fatigue, weight loss, and inflammatory disease and can be indicative of underlying kidney or liver diseases. High protein levels can indicate inflammation, cancer, dehydration, or liver/kidney disease, and low protein levels can indicate malnutrition, malabsorption, congestive heart failure, and liver/kidney disease [19]. Levels of total protein in blood serum among treatment and control mice was unchanged.

The evaluation of kidney and liver function is of great importance when determining toxicity of a certain substance or treatment. Both organs play key roles in

removing toxins from the body and maintaining a systemic equilibrium of vital nutrients needed for functioning [20]. Thus, determining the potential for hepatotoxicity and nephrotoxicity are imperative in determining the human health risk. The liver functions to filter the blood coming from the digestive tract before dissemination to the rest of the body among other metabolism and storage functions. The role of the kidney is to remove any waste from the blood to be passed in the urine and to maintain hydration and electrolyte levels [21]. Treatment group YE (.125 mg/ml anti-PSMA-MWCNT + Microwave treatment) was found to have consistently high creatinine, AST, and total protein levels, all suggesting toxicity in the liver and kidneys of exposed mice. No other treatment groups appeared to have shown levels of these biomarkers that may be indicative of toxicity. However, groups YB (Microwave only), YF (.5 mg/ml anti-PSMA-MWCNT No Microwave), and YG (.5 mg/ml plain MWCNT + Microwave) had higher AST levels which may suggest impaired liver function. It is unclear why treatment group YE elicited such a response in the mice, yet groups YF and YG only showed signs of liver damage even though a greater amount of MWCNT's were used for treatment.

Investigating gene expression can reveal which genes are turned on or off in a specific cell type and can be used to quantify the gene's relative expression. The genes studied are described in table 3. These genes play important roles in the immune and inflammatory response. Measurement of these genes in brain, liver, and kidney tissues may provide information about whether a gene is overexpressed or under-expressed in treated groups.

Gene IL1B was not expressed for the treatment or control groups in any of the tissues. Genes IL6, PTSG2, and TNF were down regulated for all treatment groups in the

brain, liver, and kidney tissues. The gene of greatest interest was NF_KB which was highly expressed in all tissues for each treatment group. The magnitude of expression compared to the control suggests a significant upregulation in the expression of this inflammatory response gene. NF_KB is a transcription regulator that is activated by various cellular stimuli such as cytokines, oxidant-free radicals, and bacterial or viral products. Activated NF_KB stimulates the expression of genes involved in a wide variety of biological functions including response to infection. Inappropriate activation of NF_KB has been associated with a number of inflammatory diseases [22]. Upregulation of this gene indicates a localized effect on these tissues that could be due to a number of cytotoxic factors including exposure to CNT-Ab plus microwave radiation.

Conclusion

In conclusion, group YE was found to have consistently high blood serum creatinine, AST, and total protein levels indicative of potential liver and kidney impairment. No other treatment groups were determined to have impaired kidney function; but groups YB, YF, and YG had higher AST levels which may suggest impaired liver function. Investigation of gene expression revealed an overexpression of the NF_KB gene, which may impact the inflammatory response pathway. These results suggest that exposure to treatment may induce hepatotoxicity and nephrotoxicity. Future studies will involve the analysis of brain, liver, and kidney histopathology to fully understand the toxicological effects of CNT-Ab exposure.

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Disclosure statement

The authors state no conflicts of interest.

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