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Assessment of the toxicological effects of CNT-Ab in mice followed by microwave hyperthermia 14 days post treatment.

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Assessment of the toxicological effects of CNT-Ab in mice followed by microwave hyperthermia 14 days post treatment.

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in *The Department of Biology.*

By
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ABSTRACT

Serious side effects and treatment resistance are the main impediments to successful cancer therapy. A variety of nanoparticles have been used for localized, site-specific treatment that prevent or circumvent these impediments. Microwaves alone have been previously used for thermal ablation of various tumors, setting precedence for their successful use in live organisms. In cell culture studies, it has been observed that multiwall carbon nanotubes (MWCNTs) instantly and efficiently absorb microwaves, causing hyperthermia of cells in direct contact with them with unnoticed harm to other cells. It is hypothesized that a treatment can be developed based on a safe microwave heating schedule for the selective ablation of tumor cells in vivo using MWCNT-ab conjugates as a targeting medium and mice as animal models. This was accomplished by conjugating MWCNTs with anti-PSMA antibodies (ab) in order to induce the selective uptake of MWCNTs by PC3 cells, known to significantly over-express PSMA antigens in comparison to non-cancerous cells. This study sought to elucidate any toxicological effects by the characterization of MWCNT-ab distribution, clearance, and toxicology in mice following microwave hyperthermia. This was assessed through clinical chemistry and analyzing gene expression in the brain, liver, and kidney. Results seem to suggest that exposure to MWCNT-Ab cause injury to the liver and kidney in higher doses. Blood serum markers of AST and creatinine were significantly elevated in some of the treatment groups. Similarly, upregulation of IL6, NFkB, PTGS2, and TNF-α in brain, liver and kidney tissues was observed for some of the MWCNT-Ab exposed mice. The results are consistent with our initial assessment that MWCNT-Ab are safe at low doses and display increasing toxicity as the concentration of MWCNTs increase. In conclusion, exposure to MWCNT conjugated antibody and microwave may produce inflammatory response in the brain, liver and kidney of mice.

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Introduction

As part of a cell's life cycle, cells grow, divide, and undergo programmed cell death. This enables cells to maintain proper characteristics and not stray into inappropriate locations. Mutations occur from errors in replication and environmental factors. Mutations that cause alterations of cell growth, cell proliferation, or DNA damage response can lead to the development of cancer [1]. Cancer is a genetic disease that arises in somatic cells causing them to proliferate abnormally and to invade and colonize areas reserved for other cells. In order for

cancer cells to proliferate without control, the cell undergoes changes that permit it to carry on dividing. Typically, this division would stop. Instead, the cell avoids cell death, displace normal neighbors, and attracts blood supply to nourish the growing tumor. To metastasize it must get in and then back out of blood or lymph vessels then survive and proliferate in the new sites [2]. Further, cancer cells exhibit a reduced dependence on signals from other cells for survival, growth, and division. For example, a mutation in a *Ras* gene can cause an intracellular signal for proliferation without presence of the extracellular signal. Cancer cells can survive conditions that would cause most cells to initiate apoptosis and this is often because of mutations to genes that regulate apoptosis [1].

Surgery, chemotherapy, and radiation are considered the three pillars of cancer treatment. However, serious side effects and treatment resistance are the main impediments to successful therapy. Surgery is effective against localized, nonhematological cancer, but it is ineffective once metastasis has occurred [1]. Radiation is a localized treatment that damages cells' genetic information making it impossible for them to grow and divide. Since cancer cells are undergoing cell division at a faster rate than healthy cells, they will be affected more than normal cells. Late stage cancers require high doses of radiation which can damage healthy tissue and limit the effectiveness of this treatment [1]. Chemotherapy broadly targets any rapidly dividing cells and is associated with severe side effects. In addition to increased proliferation, cancer cells are genetically unstable. This leads to increased mutations which is one of the factors that makes cancer so difficult to treat. No single treatment is likely to work in every patient or even every cancer cell in a single patient because the receptors of the cell are likely to change as the cell mutates.

Prostate cancer is the second leading cause of cancer death for men in many parts of the world. Current treatment is a radical prostatectomy and external beam radiotherapy. These are effective for localized prostate cancer, but many patients recur with increasing levels of prostate-specific antigen. Prostate membrane antigen (PSMA) is located on the short arm of chromosome 11 which is rarely deleted in the mutations that lead to prostate cancer [3]. There is a correlation between increased expression and the severity of the cancer and highly expressed in poorly differentiated, metastatic, and castration-resistant prostate cancer. Furthermore, it is expressed in the neovasculature structure of other cancers. This implies that a therapy that targets PSMA could be effective against late stage prostate cancer and could possibly be used to treat other cancers as well [3].

Nanoparticles differ from bulk materials in many ways. The scaling properties and quantum effects of nanoparticles affects their chemical reactivity as well as many other properties. One of such nanoparticles, namely multiwall carbon nanotubes (MWCNTs) have such unique features. MWCNTs absorb heat in the electromagnetic radiation [4]. Microwaves offer higher penetration depth with instant localized heating when combined with MWCNTs. When functionalized through the covalent conjugation of antibodies (MWCNT-ab), they gain the potential to be used for site-directed thermal ablation. The primary concern in the use of MWCNTs is their toxicity. Their minute size allows them to penetrate cells and organelles, disrupting their normal function. They have been shown to cause tissue inflammation, ischemia in the cardiovascular system, liver toxicity, and create reactive oxygen species [5].

The proposed research seeks to validate the initial promising results by characterizing MWCNT-ab distribution, clearance, and toxicology in mice following microwave hyperthermia. Furthermore, this study aims to elucidate any toxicological side effects that may result from mice

being treated with MWCNT-ab conjugate followed by microwave. Study will be evaluated through clinical chemistry and analysis of gene expression in the brain, liver, and kidney.

Experimental Design

Approximately 4-5 weeks old male mice were assigned to 8 different treatment groups, each group containing 6 mice. As specified in table 1, treatment groups varied in CNT concentration, microwave exposure, and presence of prostate membrane antigen antibodies (anti-PSMA).

Table 1: Treatments assigned to each group of mice (Z group, two-week post-treatment).

Sample	Treatment
ZA	No Treatment
ZB	Microwave only
ZC	.125 mg/ml anti-PSMA-MWCNT (No Microwave)
ZD	.125 mg/ml plain MWCNT + Microwave
ZE	.125 mg/ml anti-PSMA-MWCNT + Microwave
ZF	.5 mg/ml anti-PSMA-MWCNT (No Microwave)
ZG	.5 mg/ml plain MWCNT + Microwave
ZH	.5 mg/ml anti-PSMA-MWCNT + Microwave

Materials and Methods

Animal study

Animals were housed at the Georgia Southern University Animal Facility (1176A Biological Sciences Fieldhouse). Mice were treated according to the principles outlined in the ILAR's (Institute or Laboratory Animal Research) Guide for Care and Use of Laboratory Animals. Our protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC Protocol Approval # I18024). Mice were injected with CNTs subcutaneously in both the right and left flank and microwaved at 150 watts for 5 seconds. They

were sacrificed two weeks post treatment. Blood was collected along with the brain, liver, kidney and other tissues for further analysis.

Quantitative Determination of Serum Total Protein

The serum total protein concentration was determined using the Thermo Scientific Pierce BCA Protein Assay Kit (Catalog#: 23227, Rockford IL). This method combines the reduction of Cu^{2+} to Cu^{1+} with colorimetric detection of the Cu^{1+} ion. A working reagent was prepared using reagents A and B in a ratio of 50:1. A volume of 2.0 mL was aliquoted into test tubes. Then, 100 µL of sample was added to each tube of reagent, the samples were mixed, and then incubated at 37º C for 1 minute. The absorbance was measured at 562 nm using a spectrometer. A standard curve was created using Bovine serum albumin (BSA) to quantify the data collected.

Quantitative Determination of Serum Albumin

The serum albumin concentration in the serum samples was determined using the Albumin Reagent Set (Pointe Scientific, Inc., Canton, MI, Cat#: A7502-1L). Bromocresol green dye was bound to albumin, and the absorbance was measured at 630 nm using a spectrometer. A standard curve was created using Bovine serum albumin (BSA) to quantify the data collected.

Quantitative Determination of Serum Creatinine

The serum creatinine concentration in the serum samples was determined using Cayman's Creatinine Colorimetric Assay (Catalog# 700460, Ann Arbor MI). This assay relies on the Jafee' reaction, wherein a yellow/orange color forms when the metabolite is treated with alkaline picrate. The rate of color development is directly proportional to the concentration of

creatinine. Absorbance was measured at 490 nm. A standard curve was used to quantify the data collected and was created using the creatinine standard solution that is included in the kit.

Quantitative Determination of Aspartate Aminotransferase (AST)

The serum concentration of aspartate aminotransferase was determined using the Pointe Scientific Liquid AST reagent set (Canton, MI, Cat#: A7561-450). The set contained two reagents labeled R1 and R2. The reagents were combined and mixed with the samples following the guidelines provided by the assay manufacturer. Briefly, a volume of 1.0 mL of the combined reagents for each sample was then pre-warmed at 37º C for 1 minute. Then, 100 µL of sample was added to each tube of reagent, the samples were mixed, and then incubated at 37º C for 1 minute. 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.

Total RNA extraction

Total RNA was isolated from mouse liver, kidney, and brain using a Qiagen RNA Isolation kit (Qiagen, 2006 Valencia, CA.) The experiment was carried out using the guidelines stated in the protocol. Approximately 20-30 mg samples were homogenized in RLT buffer. RLT denatures and inactivates RNases. The RNA is then allowed to bind to a silica-gel membrane and subsequently eluted with RNase-free water. Total RNA was quantified using the UV-VIs spectrophotometer at 260 and 280 nm absorbance and quality of the RNA was determined using gel electrophoresis. The loading buffer was mixed at a ratio of 1 volume of sample to 2-5

volumes of RNA Sample Loading Buffer. Isolated RNA was stored in a -80℃ freezer for further analysis.

Analysis of select genes

FASTA mRNA sequences of the genes were obtained via the National Center for the Biotechnology Information (NCBI). Using PrimerQuest by Integrated DNA Technologies (IDT) (Coralville, IA) forward and reverse primers (Table 2) for each of the mRNA sequences was obtained. Description of these transcripts is also shown in Table 3. cDNA for use in RT-qPCR was synthesized using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, Cat#: 1708840). cDNA was diluted with 10 mM Tris-HCl buffer (pH 8), 01 mM EDTA prior to use in the qPCR system in accordance with the manufacturer's guidelines. Gene Expression was carried out using quantitative real time polymerase chain reaction (qRT-PCR) for the genes NFkB, PTGS2, TNF-α, IL6, IL1B.

Table 2. Sequence of forward and reverse primers.

Gene	Forward Primer	Reverse Primer
PTGS2	5’-GCGGGAACACAACAGAGTAT-3’	5’-GGACAGCCCTTCACGTTATT-3’
TNF-α	5’-ACAAGGCTGCCCCGACTAT-3’	5’-CTCCTGGTATGAAGTGGCAAATC-3’
IL1B	5’-TACCTATGTCTTGCCCGTGGAG-3’	5’-ATCATCCCACGAGTCACAGAGG-3’
NFkB	5’-AGCAGGATGCTGAGGATTCTG-3’	5’-GGCAACTCTGTCCTGCACCTA-3’
IL6	5’-GGAGTTTGTGAAGAACAACT-3’	5’-CTAGGGTTTCAGTATTGCTC-3’

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.

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.
NFKB	NFKB 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.
PTSG2	This gene encodes the inducible isozyme PTSG2. It is regulated by specific stimulatory events and is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis.
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.

Results

Quantitative Determination of Serum Total Protein

A standard curve of absorbance vs. concentration of bovine serum albumin (BSA) was generated. This standard curve showed a strong linear relationship demonstrated by an r-squared value of 0.9924. The linear equation generated from this linear relationship was found to be $y = 0.0803x - 0.0515$. This relationship is summarized in Figure 3. This generated equation was used to calculate concentrations of serum albumin in the mice samples.

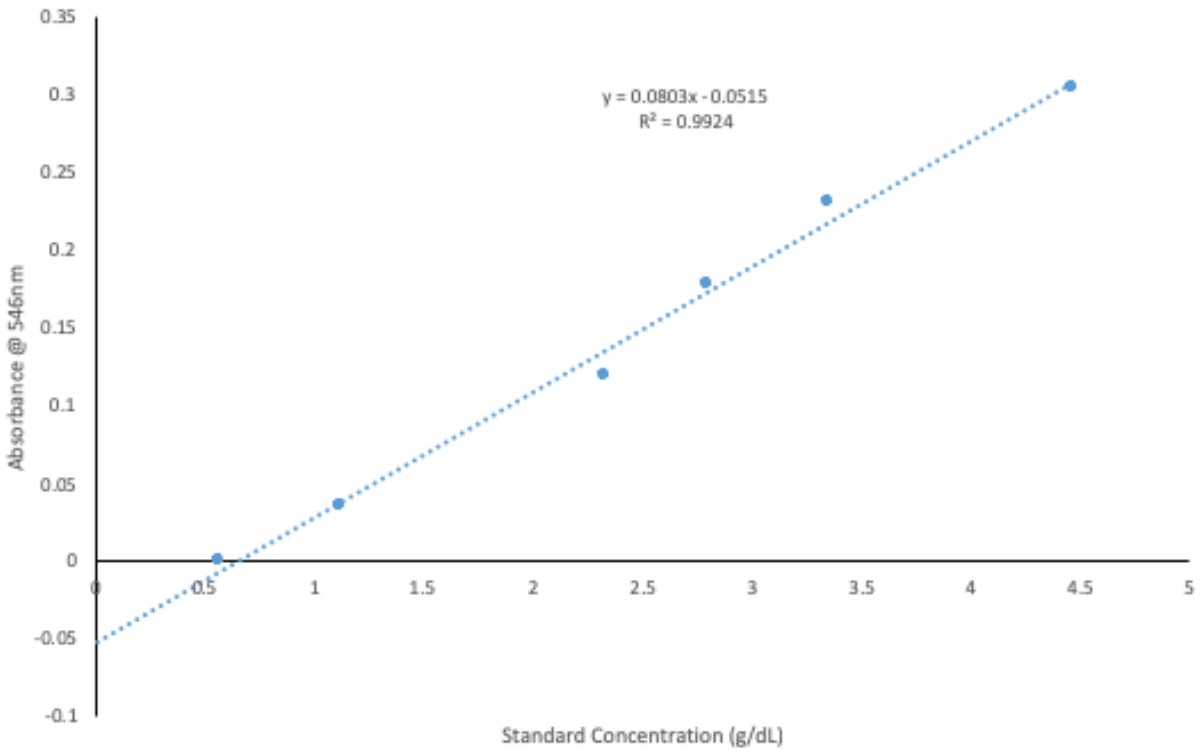

Figure 1. Standard curve of absorbance vs. concentration for bovine serum albumin (BSA).

Through the analysis of serum total protein levels in mice using the standard curve, it was found that there was no statistically significant difference in total protein concentration between groups as determined by one-way ANOVA $F(7,40) = 1.982$, $p = .08$. Each group's data is summarized in Figure 2. The standard error of each mean measurement is displayed in Figure 2.

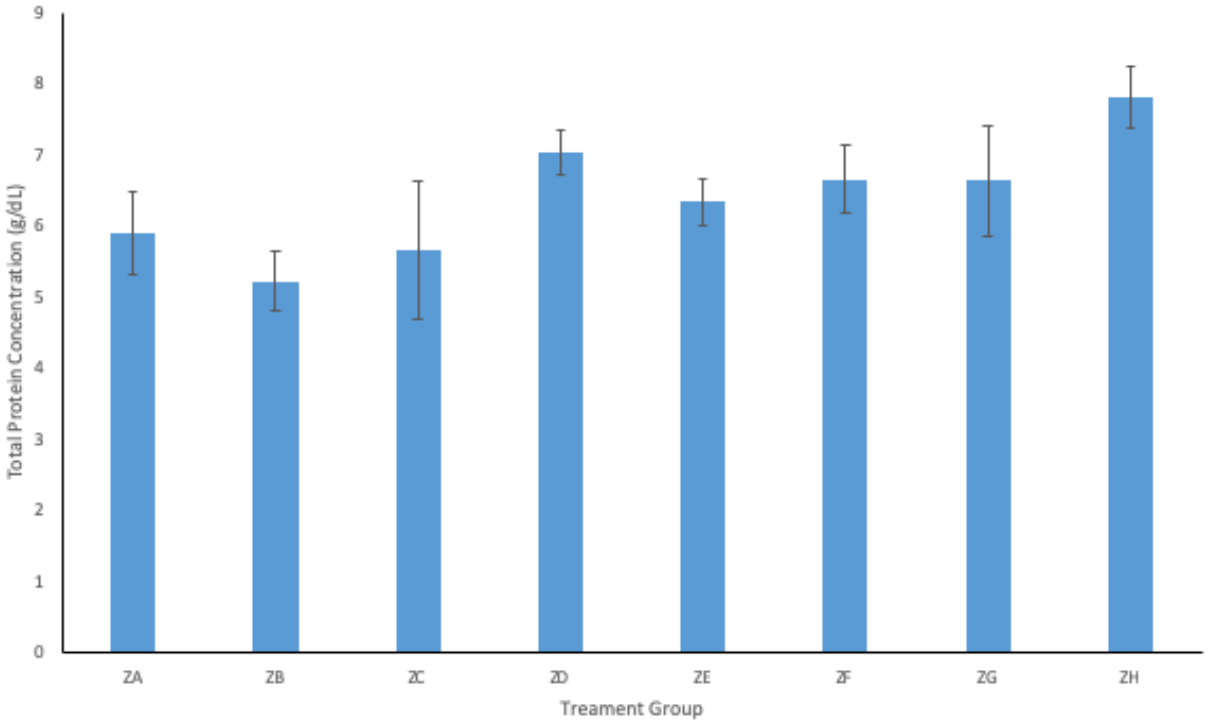

Figure 2. Mean total protein concentration from serum of mice in all treatment groups. Treatment groups included: ZA (No Treatment), ZB (Microwave only), ZC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), ZD (.125 mg/ml plain MWCNT + Microwave), ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave) and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Quantitative Determination of Serum Albumin

A standard curve of absorbance vs. concentration of bovine serum albumin (BSA) was generated. This standard curve showed a strong linear relationship demonstrated by an r-squared value of 0.9837. The linear equation generated from this linear relationship was found to be y =

0.8414x – 0.0146. This relationship is summarized in Figure 3. This generated equation was used to calculate concentrations of serum albumin in the mice samples.

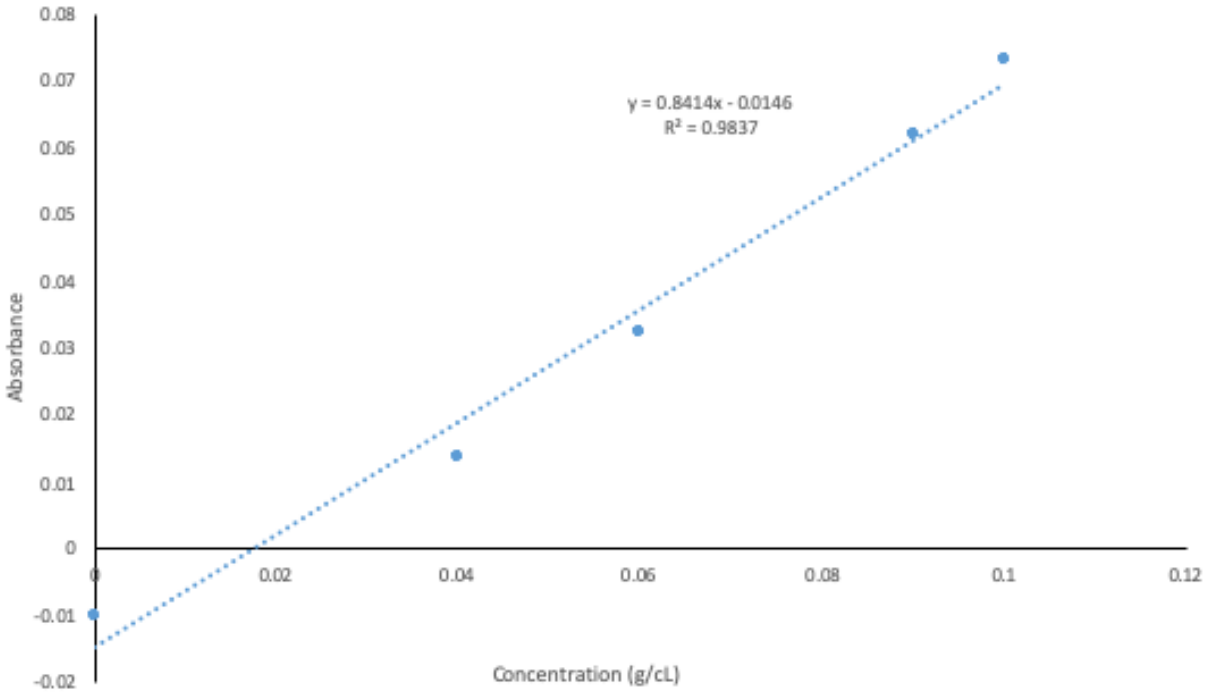

Figure 3. Standard curve of absorbance vs. concentration for bovine serum albumin (BSA).

Through the analysis of serum albumin levels in mice using the standard curve, it was found that there was no statistically significant difference in total protein concentration between groups as determined by one-way ANOVA $F(7,40) = 1.607$, $p = .16$. Each group's data is summarized in Figure 4. The standard error of each mean measurement is displayed in Figure 4.

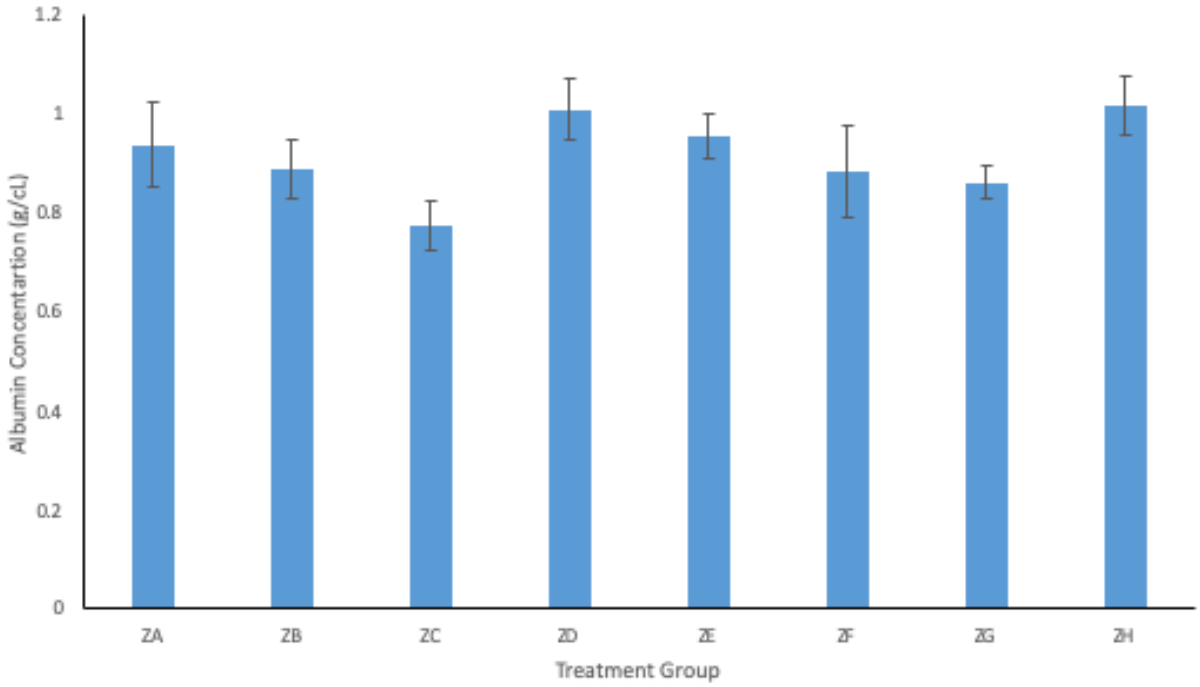

Figure 4. Mean albumin concentration from serum of mice in all treatment groups. Treatment groups included: ZA (No Treatment), ZB (Microwave only), ZC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), ZD (.125 mg/ml plain MWCNT + Microwave), ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave) and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Quantitative Determination of Serum Creatinine

A standard curve of absorbance vs. concentration of creatinine was generated. This standard curve showed a strong linear relationship demonstrated by an r-squared value of 0.9885. The linear equation generated from this linear relationship was found to be $y = 0.0031x – 0.0111$. This relationship is summarized in Figure 5. This generated equation was used to calculate concentrations of serum albumin in the mice samples.

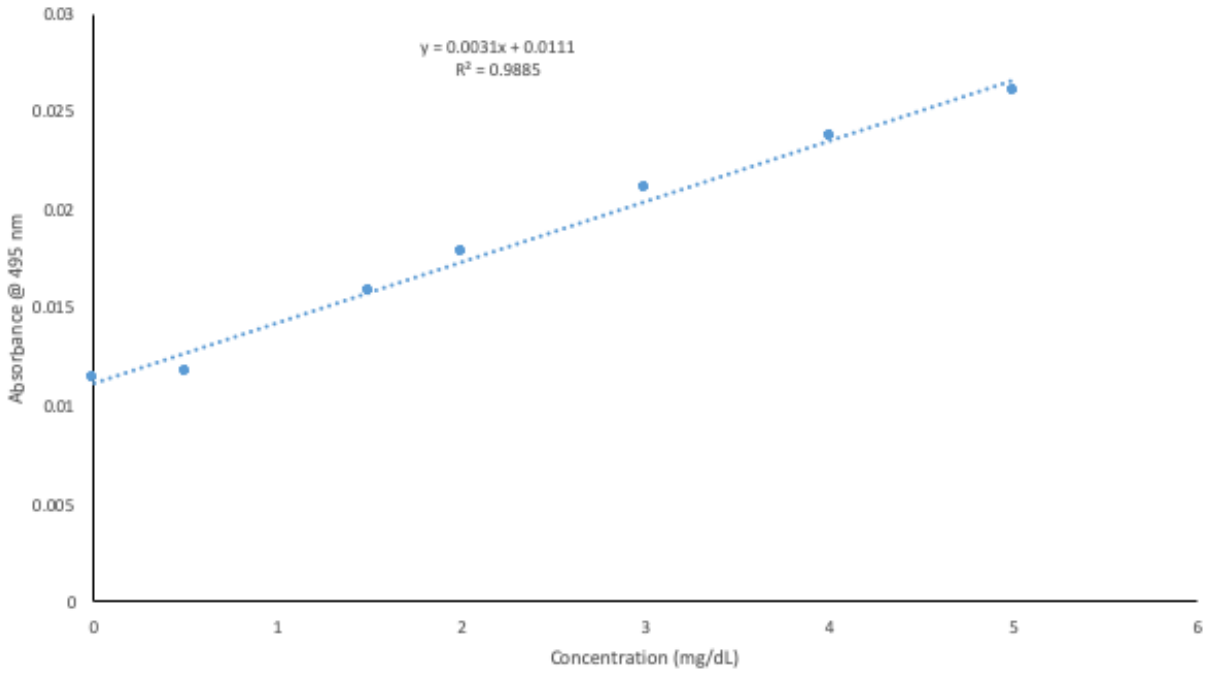

Figure 5. Standard curve of absorbance vs. concentration for creatinine.

Through the analysis of serum creatinine levels in mice using the standard curve, it was found that there was a statistically significant difference in creatinine concentration between groups as determined by one-way ANOVA $F(7,32) = 4.431$, $p = .0015$. Each group's data is summarized in Figure 6. The standard error of each mean measurement is displayed in Figure 6. Treatment groups ZB, ZC, and ZD showed no increase in creatinine compared to controls. However, groups ZE, ZF, ZG, and ZH all showed significantly elevated levels of creatinine.

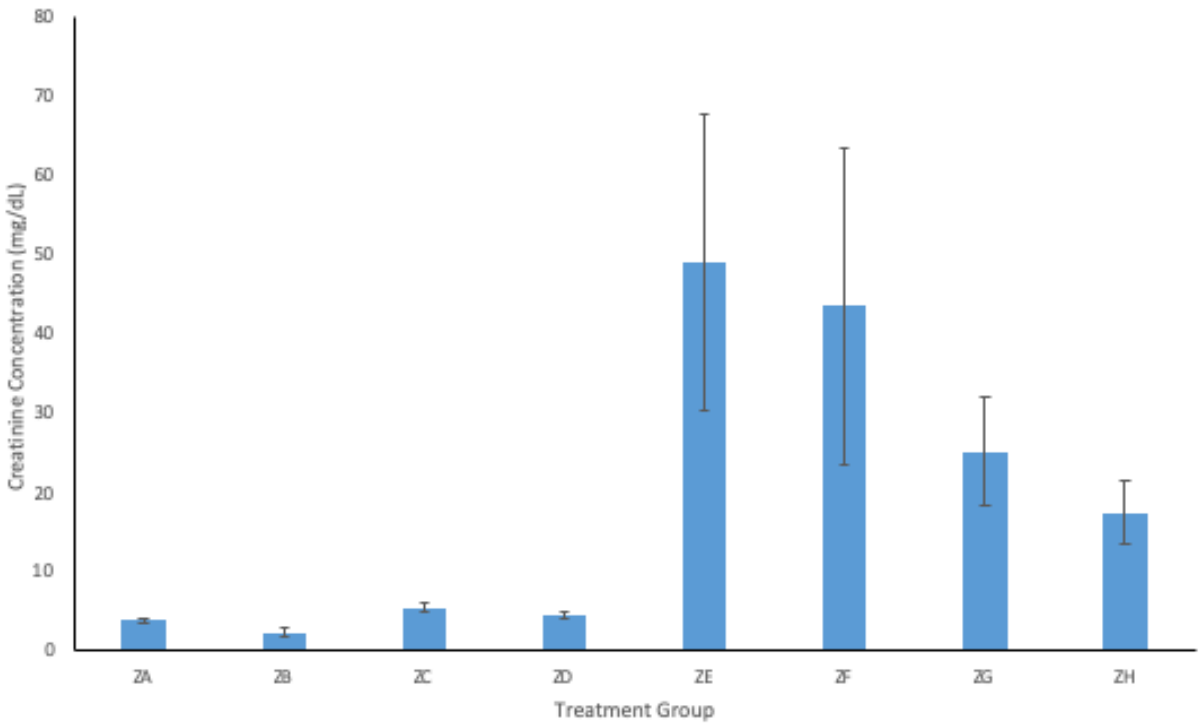

Figure 6. Mean creatinine concentration from serum of mice in all treatment groups. Treatment groups included: ZA (No Treatment), ZB (Microwave only), ZC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), ZD (.125 mg/ml plain MWCNT + Microwave), ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave) and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Quantitative Determination of Aspartate Aminotransferase (AST)

There was a statistically significant difference in AST activity between groups as determined by one-way ANOVA $F(7,48) = 2.359$, $p = .03$. Each group's data is summarized in Figure 7. The standard error of each mean measurement is displayed in Figure 7. Treatment groups ZB, ZC, ZD, ZF, and ZG showed no increase in AST activity compared to controls. However, groups ZE and ZH showed increased AST activity.

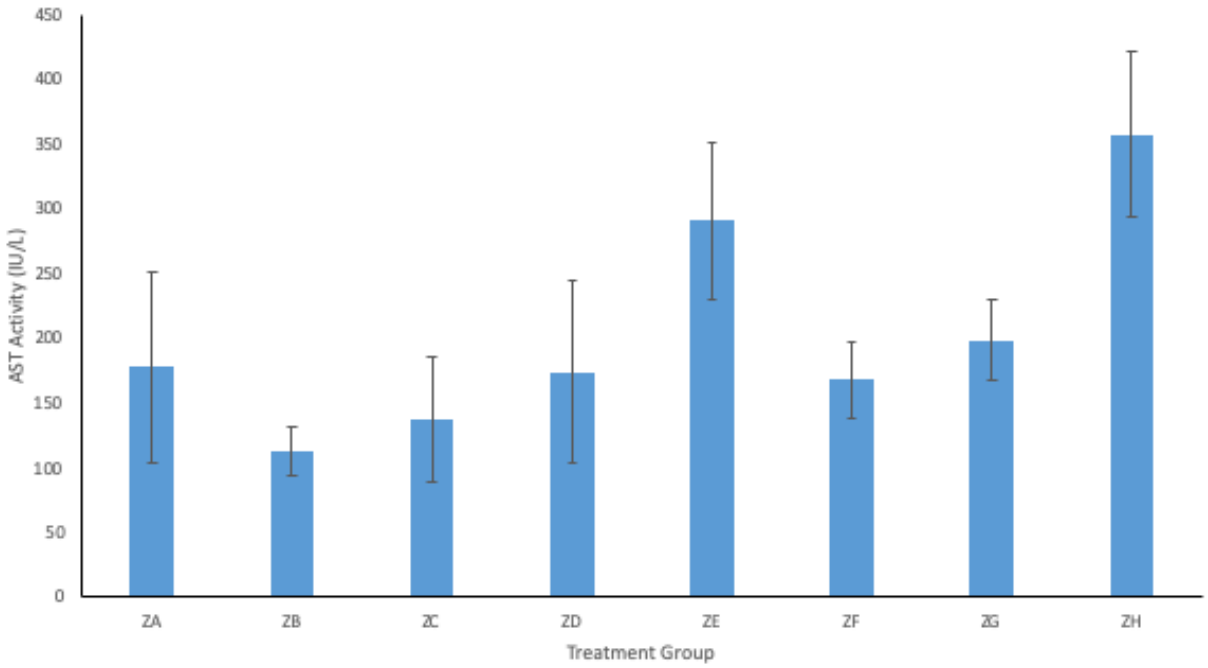

Figure 7. Mean AST activity from serum of mice in all treatment groups. Treatment groups included: ZA (No Treatment), ZB (Microwave only), ZC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), ZD (.125 mg/ml plain MWCNT + Microwave), ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave) and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Analysis of select genes in the liver

Analysis of relative gene expression in the liver is depicted in Figure 8. IL1B expression was downregulated in all treatment groups. IL6 expression was upregulated in ZC and ZG treatment groups and unchanged in all other groups. The expression of NF-$_K$B was unchanged across treatment groups. The expression of PTGS2 was upregulated in the ZG group and was unchanged in all other treatment groups. The expression of TNF-α was downregulated in the ZC and ZE treatment groups, and it was upregulated in the ZC, ZG, and ZH groups.

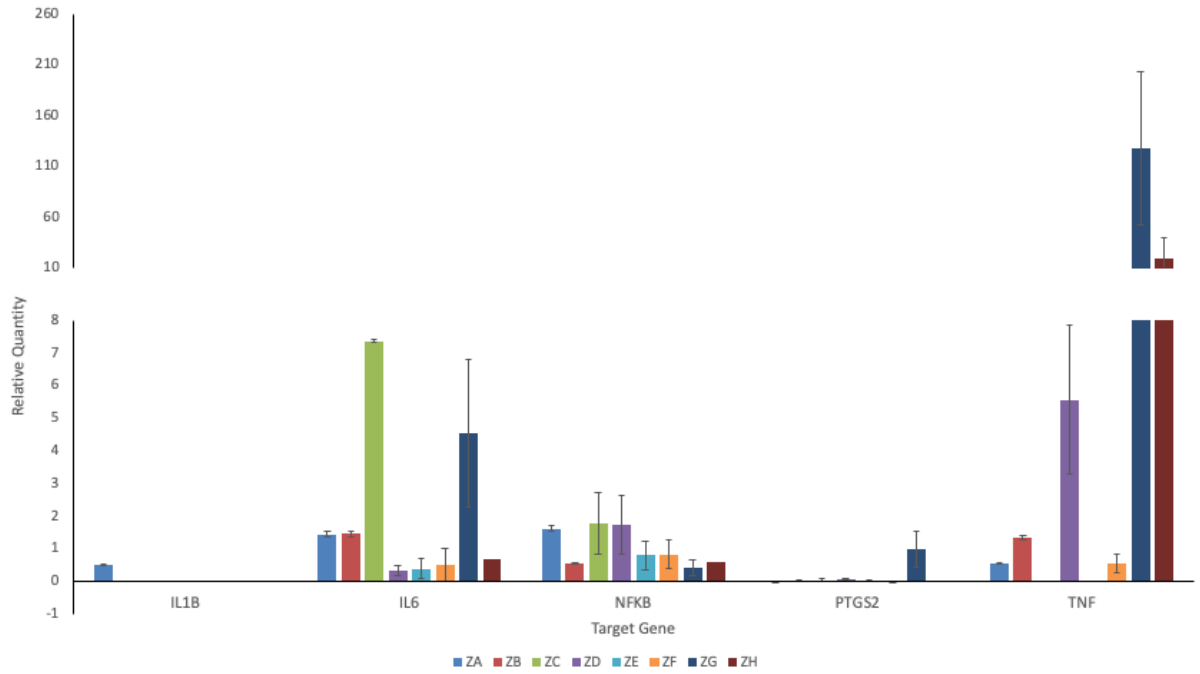

Figure 8. Liver relative gene expression in all treatment groups. Treatment groups included: ZA (No Treatment), ZB (Microwave only), ZC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), ZD (.125 mg/ml plain MWCNT + Microwave), ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave) and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Analysis of select genes in the brain

Analysis of relative gene expression in the brain is depicted in Figure 9. IL1B expression was unchanged in all treatment groups. IL6 expression was downregulated in ZB, ZD, ZG, and ZH treatment groups. The expression of NF-$_K$B up regulated in the ZH treatment group and unchanged in all other treatment groups. The expression of PTGS2 was upregulated in the ZB and ZC groups and was unchanged in all other treatment groups. The expression of TNF-α was unchanged in all treatment groups.

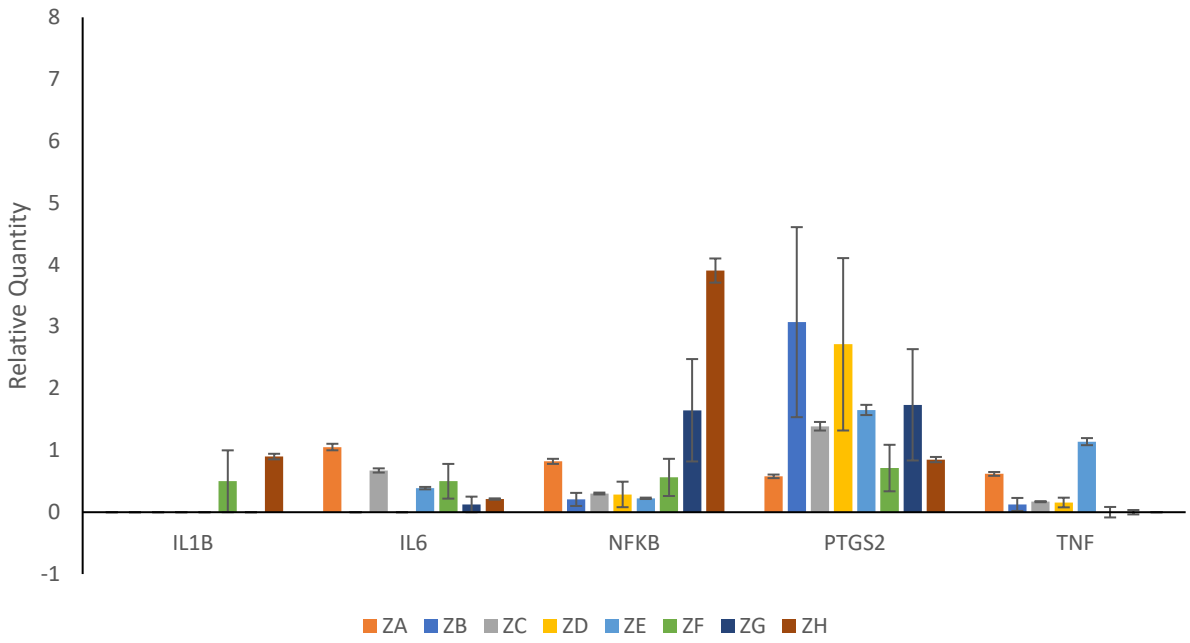

Figure 9. Brain relative gene expression in all treatment groups. Treatment groups included: ZA (No Treatment), ZB (Microwave only), ZC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), ZD (.125 mg/ml plain MWCNT + Microwave), ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave) and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Analysis of select genes in the kidney

Analysis of relative gene expression in the kidney is depicted in Figure 10. IL1B, IL6, PTGS2, and TNF-α expression was unchanged in all treatment groups. The expression of NF-$_K$B up regulated in the ZB, ZC, ZD, ZF, ZG, and ZH treatment groups and unchanged in the ZE group.

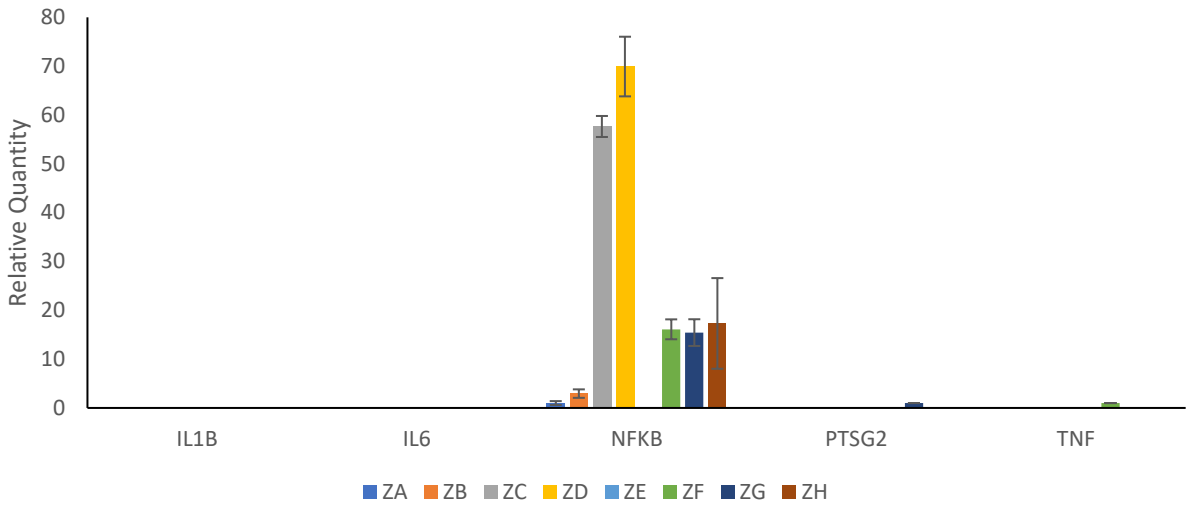

Figure 10. Kidney relative gene expression in all treatment groups. Treatment groups included: ZA (No Treatment), ZB (Microwave only), ZC (.125 mg/ml anti-PSMA-MWCNT + No Microwave), ZD (.125 mg/ml plain MWCNT + Microwave), ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave) and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave).

Discussion

Most current anticancer agents do not greatly differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects. This greatly limits the maximum allowable dose of the drug. In addition, rapid elimination and widespread distribution into targeted organs and tissues requires the administration of a drug in large quantities, which is not economical and often results in undesirable toxicity [6]. Studies have consistently demonstrated PSMA expression in all types of prostate tissue and increased PSMA expression in cancer tissue. There is demonstrated a correlation between this expression and severity of cancer. PSMA seems to be expressed in other cancers, more specifically in the neovasculature associated with these

cancers [2]. Microwaves have previously been used for tumor ablation of various tumors, including those of the lung, liver, and bones, setting therefore precedence for their use in live organisms. Microwaves offer significantly higher penetration depth, with instant localized heating when combined with multiwall carbon nanotubes. Zebrafish-PC3 xenograft embryos exposed to MWCNTs plus microwaves produced necrosis of PC3 cells [7]. The present research seeks to assess the toxicity involved in using MWCNTs conjugated to PSMA antibodies as a method to deliver site-specific treatment for prostate cancer.

Proteins are the building blocks for many components of cells and albumin is a globular protein synthesized by the liver. Decreased levels of total protein or albumin may indicate severe liver disease where the liver is unable to synthesize the protein, or it may indicate severe nephrotic syndrome where excessive amounts of proteins are being excreted by the kidneys [8]. Serum levels of albumin and total protein were studied to evaluate the hepatoxicity and nephrotoxicity caused by CNTs. It was determined that exposure to CNTs or microwaves had no significant effect on levels of albumin or total protein.

Phosphocreatine is a phosphorylated creatine molecule that is used as a reserve energy supply in the skeletal and cardiac muscles. Creatinine is a waste product formed when phosphocreatine is mobilized to replenish adenosine triphosphate levels [9]. The kidneys play an essential role in removing waste from the bloodstream so elevated creatinine in the ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave), ZF (.5 mg/ml anti-PSMA-MWCNT No Microwave), ZG (.5 mg/ml plain MWCNT + Microwave), and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave) groups could indicate that renal impairment had occurred. Exposure to high doses of CNTs have been reported to be toxic [4], thus supporting elevated creatinine levels seen in the ZE, ZF, ZG, and ZH exposure groups. It is also likely that the elevated creatinine is due to necrosis of muscle tissue at the site of CNT injection which would

release creatinine into circulation [4]. This would imply that the elevated creatinine is not due to renal failure.

Aspartate aminotransferase (AST) is an enzyme that catalyzes the transfer of an amine group from L-aspartate to 2-oxoglutarate. AST is found in many cells throughout the body but in mammals it is concentrated in the liver and heart. When these tissues are damaged, AST is released into the bloodstream and can be measured to determine liver damage [10]. The ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave) group was the only group that exhibited an increase in serum levels of AST suggesting that CNTs are less likely to cause severe damage to hepatic tissues in low doses.

Analysis of gene expression is more sensitive than the clinical chemistry assays and it provides an opportunity to understand what is occurring at a molecular level. CNTs are known to cause transient tissue inflammation [5]. While the exact mechanism whereby nanoparticles induce pro-inflammatory effects is not known, it has been suggested that they create reactive oxygen species, and thereby modulate intracellular calcium concentrations, activate transcription factors, and induce cytokine production [4]. Transcription factors play important roles in host defense and cell survival. The DNA-binding activity of these transcription factors is rapidly induced in mammalian cells by a variety of cytokines, inducing transcription of several genes involved in inflammation and apoptosis [11].

Hepatic tissues showed the largest inflammatory response in response to CNT treatment. IL6 was upregulated in the ZC (.125 mg/ml anti-PSMA-MWCNT No Microwave) and ZG (.5 mg/ml plain MWCNT + Microwave) groups, and TNF-α was upregulated in in the ZD (.125 mg/ml plain MWCNT + Microwave), ZG (.5 mg/ml plain MWCNT + Microwave), and ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave) groups. The response appears to increase in a dose dependent manner with ZG and ZH being significantly more upregulated than the ZC group. This finding seems to support the results from

the AST assays and indicates that CNTs may cause low amounts of hepatic inflammation in low doses and increasing hepatoxicity at higher doses.

The kidney showed a significant upregulation of the NFkB gene in every treatment group except ZB (Microwave only) and ZE (.125 mg/ml anti-PSMA-MWCNT + Microwave). High doses of CNTs seem to lead to upregulation of the NFkB gene and an increase in serum creatinine. It appears that CNTs may cause low amounts of renal inflammation in low doses and increasing nephrotoxicity at higher doses. This is consistent with what has been found in other studies [4]. The outlier is the ZE group which received a low dose of CNTs and demonstrated no inflammatory response yet had the highest levels of serum creatinine. The high levels of creatinine may be explained by skeletal muscle necrosis at the site of injection.

The brain demonstrated an upregulation of NFkB in the ZH (.5 mg/ml anti-PSMA-MWCNT + Microwave) group as well as an upregulation of PTGS2 in the ZB (Microwave only) and ZD (.125 mg/ml plain MWCNT + Microwave) groups. CNTs have been shown to have the ability to migrate across the blood-brain barrier neurons [4]. However, the fact that PTGS2 was upregulated in the ZB group indicates that it is possible the inflammation was not due to CNT treatment but to microwave exposure instead. The increase in inflammatory response is much less than what was observed in the liver and kidney.

Conclusion

In summary, exposure to CNT conjugated antibody and microwave produce inflammatory response in the brain, liver and kidney of mice. Results seem to suggest that exposure to CNT-Ab cause injury to the liver and kidney in higher doses. Blood serum markers of AST and creatinine were significantly elevated. Similarly, upregulation of IL6, NFkB, PTGS2, and TNF-α in brain, liver and

kidney tissues was observed. The results are consistent with our initial assessment that CNT-Ab are safe at low doses and display increasing toxicity as the concentration of CNTs increase. In the future, analysis of brain, liver and kidney histopathology will be carried out.

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Disclosure statement

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