Protein Biomarkers of Ionizing Radiation and Standardization by MALDI MS/MS and Quantification

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And hereby certify that, in their opinion, it is worthy of acceptance.

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2D – 2-dimensional

2-HED – 2-hydroxyethyl disulfide

ACN – Acetonitrile

ACTH – Adrenocorticotropic hormone

Ambic – Ammonium bicarbonate

APS – Ammonium persulfide

BCA – Bovine carbonic anhydrase

C7BzO – 3-(4-Heptyl) phenyl-3-hydroxypropyl) dimethylammoniopropanesulfonate

CHAPS – 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

CHCA – α-Cyano-4-hydroxycinnamic acid

CRP – C-reactive protein

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

EIC – Extracted ion chromatogram

ELISA – Enzyme-linked immunosorbent assay

GI – Gastrointestinal

IAA – 2-Iodoacetamide

IEF – Isoelectric focusing

IL-6 – Interleukin 6

IR – Ionizing radiation

LET – Linear energy transfer

LINAC – Linear particle accelerator

LPS – Lipopolysaccharide

MALDI TOF – Matrix-assisted laser desorption ionization time-of-flight

MS – Mass spectrometry

NCBI – National Center for Biotechnology Information

Nd-YAG – Neodymium yttrium aluminium garnet

NHP – Non-human primates

OPLS-DA – Orthogonal projection to latest structures discriminant analysis

PCA – Principal components analysis

PKM – Pyruvate kinase muscle isozyme

PLS-DA – Partial least squares discriminant analysis

RCF – Relative centrifugal force

RIPA – Radioimmunoprecipitation assay buffer

ROC – Receiver operating characteristic

ROS – Reactive oxygen species

SAA – Serum amyloid A

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

TBI – Total body irradiation

TCA – Tricarboxylic acid

TEMED – Tetramethylethylenediamine

TFA – Trifluoroacetic acid

TIC – Total ion current

Tris – tris (hydroxymethyl) aminomethane

UPLC-ESI-QTOFMS – Ultra-performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry

Abstract

The overall objective of this work was to look for proteins that are up- or down-regulated following exposure to whole-body ionizing radiation (IR). The objective is to use these proteins as potential biomarkers of exposure to ionizing radiation of 2 Gy or greater which is the tolerance level for radiation sickness. Once these protein biomarkers are obtained, their antibody could be put inside a self-administered oral test strip, called a lateral flow device, to triage victims of a radiological dispersal device or nuclear detonation. Mice were radiated with 2 Gy, 4 Gy and 7 Gy and sacrificed at 1 hour, 24 hours and 72 hours. Also, mice were injected with lipopolysaccharide (LPS) endotoxin (10 μg) to induce an inflammatory response to see if this response affects the up- or down-regulated proteins. The LPS injected mice were sacrificed at 1 hour, 24 hours and 72 hours. The tongue was chosen for this work because it is a radiosensitive tissue. The final set of experiments was the generation of controlled bovine carbonic anhydrase (BCA) spike samples to demonstrate BCA identification and quantitation. Two-dimensional (2D) gel electrophoresis was used to separate the proteins extracted from the tongue samples and MALDI TOF-TOF analysis used to identify the proteins. Absorption measurements of the 2D gel was used to quantify the proteins. IR induced up-regulated or down-regulated proteins found only in the samples exposed to radiation were troponin I, skeletal, fast 2 (up-regulated), aconitate hydratase, mitochondrial precursor (down-regulated), alpha-actinin-2 (down-regulated), pyruvate kinase isozymes M1/M2 (down-regulated) and ATP synthase subunit beta, mitochondrial precursor (down-regulated).

CHAPTER 1

INTRODUCTION

1.1 Background and Significance

If a disaster occurred such as a radiological dispersal device or nuclear weapon detonation on U.S. soil, countless victims could be exposed to ionizing radiation (IR) at levels that could affect their health. Those victims that were exposed to whole body doses of 2 Gy or greater would need treatment immediately. Those victims exposed to 2 Gy would not show immediate symptoms in 6-24 hours post ionizing radiation exposure but would likely demonstrate symptoms such as vomiting after leaving the scene. But by then, it would be too late for them to receive treatment [1].

1.1.2 The Biological Effects of Ionizing Radiation

During IR exposure there are two mechanisms that induce radiation damage [2]. One is direct action where a photon or other ionizing particle strikes DNA directly and produces ionization. However, this interaction is not a common way to cause radiation damage. The more common mechanism is indirect action where ionizing radiation interacts with water in the cell to generate free radicals and other reactive chemical species. The free radicals lead to the formation of reactive oxygen species (ROS) which attack molecules such as DNA and the mitochondria.

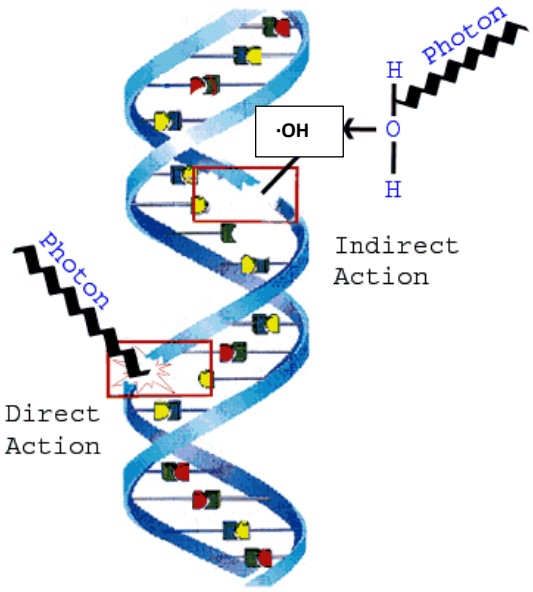


Figure 1-1. Direct and Indirect Mechanisms of Radiation Damage. Figure adapted from reference [2].

When DNA is damaged the strands are broken which causes apoptosis or mutations that could lead to cancer. When the mitochondrion is damaged it also causes apoptosis or mutations in the cells. DNA and mitochondrion damage occurs most often from organs that have rapidly growing and dividing cells which makes them sensitive to ionizing radiation. These radiosensitive organs are those of the gastrointestinal (GI) tract, such as small intestines, and bone marrow. When these organs are exposed to single high doses of radiation, acute radiation syndrome occurs. During acute radiation syndrome there are three categories of symptoms; hematopoietic, gastrointestinal and neurovascular [3].

The hematopoietic symptoms occur when the bone marrow is exposed to ionizing radiation. At total body irradiation (TBI) doses of 2 to 3 Gy the number of white cells is dramatically depleted, leading to neutropenia. At whole body doses above 2 to 3 Gy the number of red blood cells and platelets are decreased, resulted in pancytopenia. At doses of 3 to 8 Gy the bone marrow is

greatly damaged. The overall impact is to decrease the body's immune system's ability to fight off infection which is one way for someone to die from acute radiation exposure. Also, the reduction of platelets results in the limited ability to clot from bleeding increasing the risk of hemorrhaging.

Another symptom of acute TBI is the gastrointestinal syndrome. This happens when the GI tract is exposed to radiation doses around 6 to 8 Gy. Symptoms are diarrhea, nausea, vomiting and cramps. At higher doses diarrhea becomes bloody, shock occurs due to loss of blood, called hypovolemia, and death. These symptoms happen because the cells from the mucosal lining are too damaged to continue to divide normally so that as the mucosal lining is removed it cannot be replaced. The mucosal lining is part of the gastrointestinal wall that takes up nutrients from food. When it is damaged the body cannot absorb food and the contents of the intestine are expelled through diarrhea or vomiting. There is also dehydration, malnutrition, gastrointestinal bleeding and sepsis or inflammation throughout the body caused by infection. Because the gut is full of bacteria and fungi and when the barriers break down the infectious agents invade [3].

The last symptom of acute TBI is the neurovascular syndrome. This happens when victims received a fatal dose of over 30 Gy. By then there is an overflow of free radicals in the cells, the bone marrow and GI tract are permanently damaged, and the circulatory and nervous systems are affected. Symptoms include cerebral anoxia or loss of oxygen from the brain, loss of serum, edema or loss of fluid, loss of electrolytes from tissue leakage, severe illness, a burning sensation, vomiting, confusion, central nervous dysfunction and respiratory system collapse which results in death.

1.2 Using Protein Biomarkers to Triage Victims of IR

Victims would be exposed to different doses of ionizing radiation as a result of a radiological dispersal device or nuclear weapon detonation. Those individuals who received less than 2 Gy TBI will not need medical care since their body will not succumb to acute radiation sickness but instead recover from it. However, victims who received 2 Gy or greater will need medical care immediately to combat radiation sickness within, 6 to 24 hours. In the past, the only way to detect if someone has been exposed to radiation sickness was to see the victim vomiting or having diarrhea. But by then the sickness was progressing. The need for triage is important because the objective is to be as efficient and timely as possible in knowing who needs immediate medical care and can benefit from medical care as the local health care system is likely to be overwhelmed with victims.

One approach to triage is to use proteins as biomarkers for levels of TBI exposure. During IR exposure, proteins from radiosensitive organs will either be up- or down-regulated. Conceptually, antibodies for the protein biomarkers could be impregnated into a lateral flow device that could detect the biomarker by a simple test such as a color change. The idea is that the lateral flow device could triage the victim by rubbing the person's tongue with the test strip and based upon the color change provide immediate information on the TBI dose received by the individual. The tongue will be tested because it is one of the radiosensitive organs. The other advantage of such an approach is that swabbing the tongue is not an invasive process.

The initial idea to find proteins biomarkers of IR exposure in radiosensitive organs was from a previous experiment [4]. In that experiment, mice were treated with 3 Gy IR from the head and neck and sacrificed at 15 and 30 minutes. The IR exposed mice where compared to controls. Then their buccal mucosa was removed where their proteins were extracted from a high

pressured instrument called a Barocycler. These proteins were analyzed with matrix-assisted laser desorption ionization (MALDI) in linear mode and reflectron mode. In linear mode, proteins were tentatively identified since the MALDI instrument only performed MS. In the reflectron mode peptides were identified because MALDI could perform MS/MS. The results showed there was protein and peptide changes from IR mice in comparison to controls. In linear mode, the protein identified from 3 Gy at 15 minutes was DnaJ homolog subfamily C member 1 (63870.32 m/z) and at 30 minutes post 3 Gy IR exposure was mCG1463, isoform CRA_b (72449.25 m/z). In reflectron mode at 15 minutes post 3 Gy IR exposure transaldolase and an unnamed protein was identified and at 30 minutes a novel protein was identified.

Since there were protein changes in the buccal mucosa from IR exposure, another experiment was conducted to see if there were protein changes from other organs [4]. In that experiment, mice received TBI with a low linear energy transfer (LET) photon beam (Philips GMBH Linac 23 MV linear accelerator, Eindhoven, Holland) at doses of 1, 2 and 3 Gy and were compared to controls. Each set had 10 mice. The mice were sacrificed immediately by injecting 5 mL of 30% sucrose in the heart and paraformaldehyde which fixes tissue to prevent it from decaying. Then the heart and tongue from each mouse was harvested where they were immersed first in paraformaldehyde for one hour then immersed in 30% sucrose until equilibrium was reached. The organs were ready to be mounted on a cryotome where sections were made for histology, MALDI imaging (and MS) and liquid chromatography mass spectrometry (LCMS). The MALDI MS and LCMS results showed there were protein changes which were identified between the TBI mice and controls. These results correlated with the histology and MALDI imaging where the higher the dose the greater the tissue damage. However, the LCMS results were not reproducible thus a new method was needed to find proteins of IR exposure.

1.3 Protein Biomarkers for IR Exposure

Previous work has investigated using blood and urine to identify proteins and other biomarkers of IR exposure. In one study by Blakely, non-human primates were irradiated with TBI ^{60}Co γ-rays (60 cGy min-1), to doses of 1.0, 3.5, 6.5 and 8.5 Gy [5]. Their urine was collected at 12, 24, 36, 48, 60, 72 and 84 hours and the samples were prepared and run through an ultra-performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) and analyzed for proteins. The collected urine samples were prepared, and analyzed by UPLC-ESI-QTOFMS.

The mass spectral data was analyzed by MarkerLynx (Waters, Milford, MA). This program normalized the data by the total ion current (TIC) chromatogram. TIC is when all of the intensities of the masses detected are added [6]. The normalized data was then turned into a multivariate data matrix by peak picking and deisotoping which uses algorithmic equations to remove unwanted high mass isotope peaks from an isotopic cluster to show the monoisotopic peaks [7, 8]. By performing peak picking and deisotoping, data becomes less convoluted. To reduce the noise from the mass spectral data, the data was analyzed by principal components analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latest structures discriminant analysis (OPLS-DA). Specifically, OPLS-DA was used to identify potential biomarkers by comparing controls to four different levels of ionizing radiation (1, 3.5, 6.5 and 8.5 Gy) and seven post-dose times (12, 24, 36, 48, 60, 72 and 84 hours).

Once the data was corrected, it was compared to a calibration curve to quantitate the biomarkers. These standard calibration curves ranged from 0 to 35 μM and for the metabolites isethionic acid, N-acetltaurine, creatine, creatinine, adipic acid, 2'-deoxyuridine, 2'-deoxyinosine,

thymidine, N-acetylspermidine, taurine, N-hexanoylglycine, hypoxanthine, xanthosine, uric acid, xanthine, N-acetylserotonin sulfate, tyrosine, tyramine and tyrosol sulfate.

In this experiment, the group used a two-tailed Mann-Whitney *U* test. The Mann-Whitney *U* test is similar to the Student's *t*-test in that it is used to determine if there is a difference between sample groups. Unlike the Student's *t*-test, the Mann-Whitney *U* test is nonparametric and does not assume that the probability distribution is Gaussian [9]. Another type of analysis used was called the receiver operating characteristic analysis (ROC) which checked how robust the biomarker identification was for each dose and time point by comparing the concentrations of urine collected from radiated non-human primates (NHP) for each biomarker. Last, Blakely checked that the biomarkers were quantitated by the peak area integration of the extracted ion chromatogram (EIC) and external standards. EIC is when one or more mass-to-charge values representing one or more analytes of notice are obtained from the whole data set for a chromatographic run [10]. The total intensity within a mass tolerance boundary around a specific analyte's m/z ratio is plotted at each point in the analysis. This is useful for checking data to spot unseen analytes, to detect potential isomers or to have clean chromatograms.

The results showed that thirteen metabolites were up-regulated in urine as a result of TBI on non-human primates. The protein biomarkers were; N-acetyltaurine, isethionic acid, taurine, xanthine, hypoxanthine, uric acid, creatine, creatinine, tyrosol sulfate, 3-hydroxytyrosol sulfate, tyramine sulfate, N-acetylserotonin sulfate, and adipic acid. These biomarkers were found in non-human primates and were compared to the biomarkers from a previous experiment performed on mice and rats.

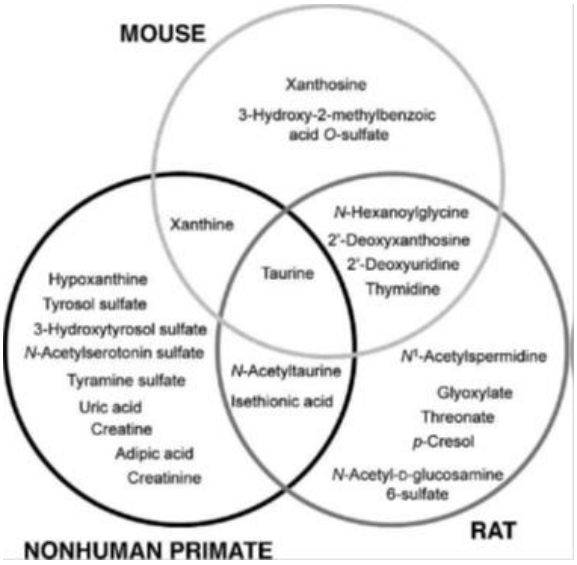


Figure 1-2. Markers of ionizing radiation in rats, mice and non-human primates taken from reference [5].

These biomarkers are not distinctive metabolites but Blakely displayed quantitative urinary changes of these metabolites post TBI. The most important changes were from up-regulated biomarkers at certain doses of ionizing radiation. For instance, at 8.5 Gy and twenty four hours, there was a distinct increase in concentration of isethionic acid, N-acetyltaurine, taurine, adipic acid, hypoxanthine, uric acid, xanthine, 3-hydroxytyrosol sulfate, N-acetylserotonin sulfate, creatinine and tyramine sulfate. Another group of metabolites changed in the range of twenty four to thirty six hours after exposure to 6.5 Gy ionizing radiation exposure in comparison to the predose values. These were isethionic acid, N-acetyltaurine, taurine, hypoxyathine, uric acid and creatinine. Blakely reported that these six metabolites are biomarkers for 6.5 to 8 Gy radiation exposure because creatine was only up-regulated at 6.5 Gy at 24 hours post radiation. Other specific metabolites were hypoxanthine because it was the only one that increased over the dose range of 3.5 to 8.5 Gy. Also, tyrosol sulfate and hydroxytyrosol were significant because they were up-regulated at 3.5 Gy, forty eight hours post-TBI dose.

The up-regulation of the afore-mentioned metabolites from ionizing radiation exposure is consistent with their production. For example, xanthine, hypoxanthine and uric acid are metabolites involved in the pure catabolism pathway. Xanthine and hypoxanthine are formed from the deamination of guanine and adenine which is greatly increased from ionizing radiation. Xanthine and hypoxanthine are known to induce mutations in DNA. This happens when adenine and guanine are deaminated thus hypoxanthine combines with cytosine while xanthine pairs with thymine. Also, both these metabolites can go through depurination which also results in forming a mutation. However, the most common cause of mutation is deamination by the nitrous reactive oxygen species (ROS) caused by ionizing radiation [5].

The metabolites involved in the purine catabolic pathway were not the only biomarkers; the ones in the taurine metabolism are taurine, N-acetyltaurine and isethionic acid. For taurine, its role is in bile acid conjugation, anti-oxidation and protection of the body by inhibiting ROS [5]. It also regulates kidney sulfur levels. Blakely hypothesized that tissue injury (due to ionizing radiation) induces excess taurine as a way of removing sulfur in the urine. Another explanation for the increase of taurine is to protect cells against injury caused by acute radiation syndrome.

The other biomarkers, creatine and creatinine, showed an increase in urine because creatinine is made by creatine and phosphocreatine; this indicates that the muscles are not able to use creatine when irradiated and instead it is excreted in the urine. Another important pathway affected by ionizing radiation is the beta oxidation pathway for gluconeogenesis. The metabolite involved and up-regulated was N-acetylserotonin sulfate which is an intermediate of melatonin; its function is to protect DNA, lipids and proteins against oxidative stress caused by radiation [5]. An increase of N-acetylserotonin sulfate is caused by rise of melatonin metabolism levels. The last pathway identified in this study that is affected by ionizing radiation is the tyrosine pathway

with the following proteins, tyramine sulfate, tyrosol sulfate and 3-hydroxytyrosol which were released in the urine as a way to remove excess tyrosine, made after ionizing radiation. The concentration of tyrosine can increase phenylalanine which goes through hydroxylation by hydroxyl radicals. These radicals cause oxidative damage to proteins.

In another study by Blakely, male mice (8–10 weeks old) were exposed to ^{60}Co γ-rays TBI over a dose range of 1 to 7 Gy [11]. Following the exposure, blood samples were collected and enzyme-linked immunosorbent assay (ELISA) was used to measure protein biomarkers, serum amyloid A (SAA), interleukin 6 (IL-6) and GADD45α. The results were analyzed by Wilks' Lambda statistics and a two-sided Student's *t*-test to find out the discriminatory power between the control and treated groups in which the values are expressed as the standard error of means (SEM). Discriminatory power is the number of animals that were classified correctly compared to the total number of animals from each comparison group. A plot of Wilks' Lambda (discrimination power) versus Dose (Gy) showed that the greater the dose, greater the discriminatory power. Protein content (pg/mL) is the dependent variable while Dose (Gy) is the independent variable, the higher the dose, the larger the content.

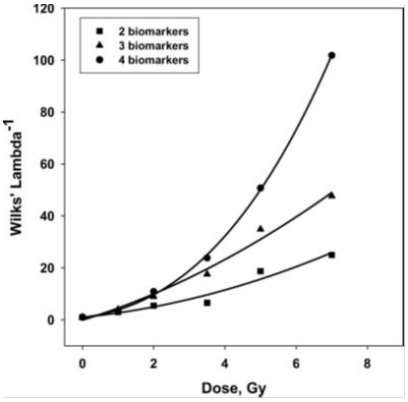


Figure 1-3. Dose response for Wilks' lambda-1 discrimination parameter taken from reference [11].

This graph shows the results of discriminant analysis for different combinations and biomarkers after 2 Gy TBI. In general, the discrimination of 2 Gy compared to a control is significant. Also, the work demonstrated that IL-6 at 4 hours contributes most to the discriminatory power followed by SAA, then GADD45α and last IL-6 at 24 hours which contributes the least in matters of multiple biomarker combinations. However, individually the discriminatory power for SAA is 87.5%, GADD45α is 100%, IL-6 at 4 hours is 93.8% and IL-6 at 24 hours is 81.3%. The discriminatory power increases at a range of 93.8-100 when there is a combination of two biomarkers while the mean value is 97.1% and standard deviation is 3.2% when there are six biomarker combinations. The best discriminatory power was when there were four combinations of three biomarkers or one combination of four biomarkers. That discriminatory power for those two scenarios was 100%.

In this work the Student's *t*-test was used to determine the differences between time and dose in each group. The Student's *t*-test results showed an increased progression of protein expressions found in mouse plasma for SAA, IL-6 and GADD45α. Specifically, the expression of SAA increased significantly in irradiated mice at 24 hours post exposure to 2 Gy or more. The concentration of IL-6 increased greatly in plasma of mice at doses greater or equal to 1 Gy, post 4 hours irradiation. For GADD45α the levels increased dramatically at 24 hours and lasted up to 48 hours post irradiation. However, those results were for only a single dose of radiation. For a range of doses, SAA and IL-6 (at 4 hours) showed important results when comparing irradiated groups for every combination of doses. For IL-6 at 24 hours there was great change after comparing irradiated groups for combinations of 1 Gy and 3.5 Gy doses and higher, 2 Gy and 7 Gy; 3.5 Gy and 7 Gy. From these results, Blakely concluded that there is a strong connection between different combinations of radiation-responsive blood protein biomarkers.

The results from the various analysis techniques correlate to the functions of these blood protein biomarkers of ionizing radiation exposure. For instance, GADD45α is a protein with multiple functions such as strand break repair and being involved in DNA replication. It is also a part of stabilizing the genome, controlling cell-cycles and their growth and cell death [11].

As for the other protein biomarkers, IL-6 was investigated because it is a cytokine that participates in a succession of actions after irradiation [11].This biomarker is a significant contributor for resistance to deadly irradiation and displayed an important effect on hematopoietic recovery post radiation exposure. IL-6 plays a role in the activation of multiple transcription factors. It is also known to have increased levels in plasma of intestinal and liver-irradiated mice which was found within 48 hours post irradiation.

The other protein biomarker SAA is an acute phase protein that is sensitive to inflammation and tissue damage. SAA is involved in significant roles with damage caused by radiation especially during acute events. This protein had the most rapid level increase out of all acute-phase proteins. The levels of inflammatory SAA response was primarily found in bone marrow cells of mice exposed to TBI, specifically at 24 hours post radiation. Thus with these results and the known functions of these proteins, Blakely proposed that SAA, GADD45α and IL-6 could be potential biomarkers [11].

The most recent work from Blakely used non-human primates that were radiated with TBI at doses of 1, 3.5, 6.5 and 8.5 Gy ^{60}Co gamma rays [12]. After these primates were exposed to radiation, their blood was collected at a time range of 6 hours to 60 days. The collected blood was run by sandwich ELISA which analyzed proteins serum amyloid A (SAA), C-reactive protein (CRP), interleukin 6 (IL-6) and Flt3 Ligand (Flt3L). These were analyzed because CRP

is a multi-subunit protein found in blood plasma where the concentration levels rise during inflammation [13]. The other protein SAA is also involved during inflammation and is concentrated primarily in the liver to recruit immune cells to the inflamed location. Normally SAA concentration levels increase during inflammation [14]. Interleukin 6 plays a role in fighting infection caused by tissue damage leading to inflammation. Flt3L is a cytokine that signals blood progenitor cells to heal wounds [15].

After these proteins were run through ELISA, they were analyzed using a two group Wilk's Lambda test. However, when only two groups were compared the Student's *t*-test was used between samples by time and dose. The Wilk's lambda and Student's *t*-test were used to check if changes in these proteins were significant.

The results showed that Flt3L levels increased from TBI non-human primates for a given dose over time [12]. At first there did not seem to be a difference between the NHP exposed to TBI and the controls because the Flt3L levels were the same at 6 hour post TBI from 1 to 3.5 Gy in comparison to the controls. However, at 8.5 Gy (6 hours post TBI) there was a significant increase in Flt3L. The Flt3L levels continued to greatly increase at day two post TBI at 6.5 and 8.5 Gy dose. By day 4 Flt3L levels rose linearly for doses of 1, 3.5, 6.5 and 8.5 Gy at day 7 the Flt3L is at its peak before declining. In general, the level of Flt3L concentration increased as the doses increased.

As for the other proteins, IL-6, SAA and CRP their concentration levels greatly increased for post TBI blood samples and peaked at 6 hours [12]. After 6 hours their levels began to decrease until returning to baseline at day 2. These results were for proteins analyzed individually. However, when these proteins were analyzed by the multivariate linear-regression as a

combination to determine the best biomarker, using all four biomarkers together was the best approach. This was determined by comparing the results for CRP alone to CRP with IL-6 or with all of the biomarkers. The combination of all protein biomarkers worked because IL-6, SAA and CRP are predicted to be used for early-phase biodosimeters since their levels peaked at 6 hours while Flt3L was seen to be the most dominant biomarker due to its concentration level peaking at seven days.

The combination of biomarkers was also used because of the concern with only using CRP or SAA alone or a combination of just these two proteins is that a false positive could happen if they are used to triage victims of TBI exposure [12]. The false positive is believed to occur because CRP and IL-6 levels increase as part of an inflammatory response which can be induced in multiple ways in addition to TBI. However, an elevation of Flt3L does not induce a false positive because it is not dependent on inflammation but from tissue damage, especially from bone marrow. To check that Flt3L does not produce a false positive, the authors also used a female mouse TBI model and when tested with stress, infection or trauma the Flt3L levels did not increase within 1 hour post radiation injury [12]. This result displayed that Flt3L was a radiation-specific biomarker.

Ghosh et al. also investigated biomarkers of ionizing radiation exposure in blood and urine [16]. Mice were put in Plexiglas boxes where they received either sham irradiation or 4 Gy and 8 Gy IR from ^{60}Co gamma rays. Then these mice were returned to their cages and were given food and water and observed for symptoms of radiation sickness such as weight loss. Blood was collected at day one or day four. After blood was collected, the mice had their organs harvested on the first or fourth days. These gastrointestinal (GI) tissue samples were prepared and blood was run by UPLC-TOF-MS.

The data from UPLC-TOF-MS was normalized then analyzed by principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) to distinguish between sham irradiated and 4 Gy and 8 Gy IR. In addition, the Madison Metabolomics Consortium Database was used for metabolite identifications. From the OPLS analysis the sham irradiated mice were compared to the IR mice and controls.

From the UPLC-ESI-TOF-MS profile that was created from the GI tissue results, tryptophan was identified as up-regulated at one day post IR exposure [16]. Also identified were glutamic acid, Cysteine-Glycine and methionine as down-regulated from one day after IR exposure. When analyzing these tissue samples using UPLC-QTOF-MS in electrospray, spermidine, and eicosenoic acid and taurocholic acid increased in 4 Gy and 8 Gy post-IR exposures. Also identified was UDP-N-acetyl-D-glucosamine that decreased after IR exposure.

The authors conclude that the IR damage of the GI tract disrupts the regulation of tryptophan [16]. As for glutamic acid the decrease is due to GI tissue damage since 80% of glutaminase activity is found in the GI tract. For Cysteine-Glycine the reduced level could be from oxidative stress caused by radiation exposure. The authors did not mention why other metabolites such as UDP-N-acetyl-D-glucosamine were down-regulated or spermidine and eicosnoic acid was up-regulated; they did note that spermidine's concentration level did increase in a previous study [17]. For taurocholate, this metabolite has been involved in causing gastric mucosal ulcers when up-regulated. The authors were able to identify seven biomarkers of radiation exposure in the GI tract. From their results, the authors concluded that detecting and identifying changes in metabolites from GI tissues could be used as biomarkers for IR exposure.

1.4 Using Tongue and Two Dimension Gel Electrophoresis to Identify Protein Biomarkers

Previous work has demonstrated that biomarkers in blood, urine, and tissue can be used to determine the level of TBI. Collection and analysis of these biomarkers is, however, invasive and time consuming as the samples require significant sample preparation. The objective of this work was to search for protein biomarkers from the tongue following TBI that could be used as an indication of TBI.

Two dimensional gel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry time-of-flight (MALDI-TOF-TOF) was used to analyze proteins from the tongue samples. The advantage of using 2D gel electrophoresis is it consistently separates proteins by pI and size. Also, when these proteins are separated, they can be analyzed by MALDI TOF-TOF for protein identification.

CHAPTER 2

EXPERIMENTAL PROCEDURE

2.1 Overview of Methods: Rationales & Explanations

Figure 2-1 shows a flow diagram of the procedures used to process and analyze the mouse tongues to search for proteins that were up- or down-regulated following exposure to whole body ionizing radiation.

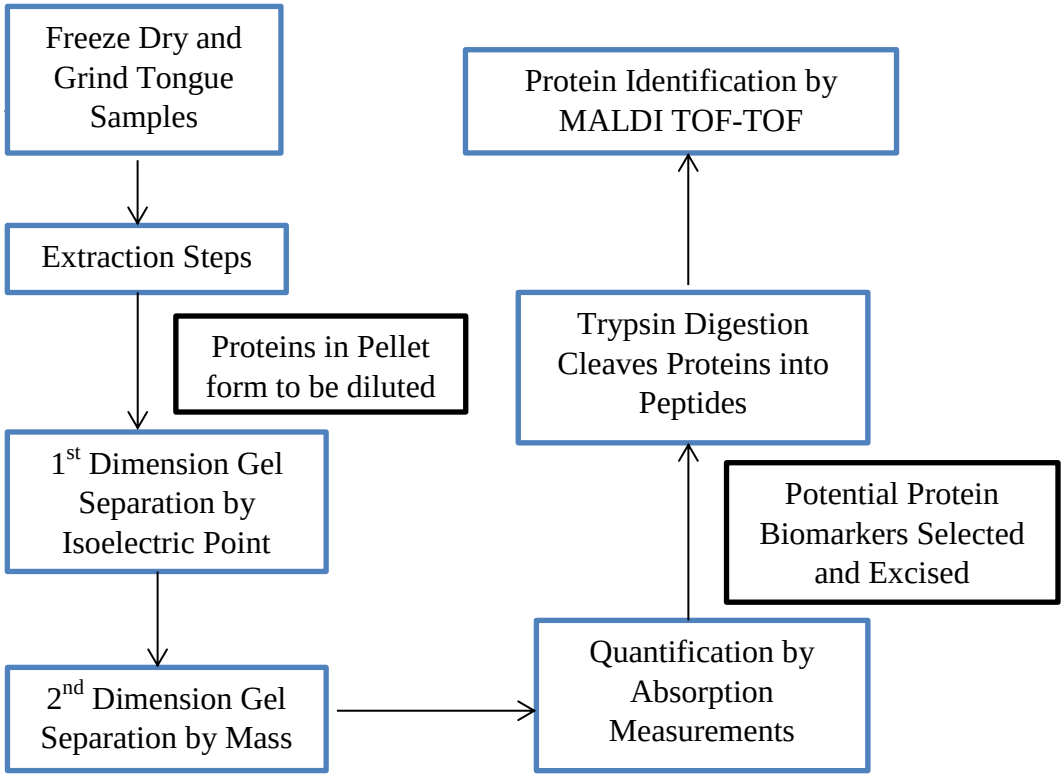


Figure 2-1. The general steps for protein identification and quantification.

2.1.1 Sample Preparation and Processing

Tongue samples were obtained from the heads of decapitated mice (see 2.2) using forceps and scissors to remove the tongues from control mice or mice subjected to LPS injection or total

body irradiation (TBI). LPS was used because it is an endotoxin that induces an inflammatory response in the epithelial tissues in mice [18]. This would be similar to a person having gingivitis. Mice injected with LPS were examined because Homeland Security was concerned that a lateral flow device with an antibody of a protein biomarker would induce a false positive due to gum disease.

These samples were placed in a container filled with liquid nitrogen to preserve the tongue and prevent protein degradation. The freeze dried tongue sample was ground into a powder with a mortar and pestle to facilitate subsequent protein extraction. The extraction solution was composed of tris-phenol in extraction media containing mercaptoethanol. The mercaptoethanol degrades proteins by cleaving the disulfide bridges making it easier for MALDI TOF-TOF analysis. Each sample was put through two extraction steps and a back-extraction. The purpose of the extraction steps was to extract as much protein as possible to precipitate a pellet for 2D gel electrophoresis. Finally, the extracted proteins were washed with ammonium acetate in methanol with dithiothreitol (DTT) twice and 80% acetone with DTT to induce more protein precipitation. DTT acts to degrade proteins in a manner similar to mercaptoethanol.

2.1.2 Protein Dilution and Passive Rehydration

Before tongue samples could be run on two dimensional (2D) gel electrophoresis they had to be diluted with isoelectric focusing buffer (IEF) to equal total protein concentrations. It is also necessary to avoid attempting the electrophoresis with excess protein concentration, as the proteins would not separate adequately and there would be streaking in the gel. To determine the required dilution with IEF buffer, the samples and a standard were pipetted in a microarray and washed with a fluorescent agent called EZQ. This microarray was scanned by the Fujifilm FLA

5000 instrument (FUJIFILM Life Science, Tokyo, Japan) and analyzed by software Multi Gauge (FUJIFILM Life Science, Tokyo, Japan) which measures the total protein concentrations. From there the total protein concentration for each sample was calculated [19]. Ampholytes pH 3-10 (2.5 µL), glycerol (6.0 µL) and 2-hydroxyethyl disulfide (2-HED) (5.5 µL) and bromophenol blue (0.25 µL) was added the diluted sample and vortexed. Ampholytes establish the pH gradient for the separation, glycerol improves the adherence of the sample to the 1D strip, 2-HED is a reducing agent that minimizes oxidation during the 1D separation, and bromophenol blue provides a color indication that the separation is complete. The vortexed sample (200 µL) was pipetted on to the equilibration rack where the 1D strip was placed on top and filled with mineral oil to prevent dehydration. The sample was allowed to undergo passive rehydration overnight. This was done so that the proteins from the diluted sample would have time to penetrate the dry 1D strip to rehydrate it and be ready for the second dimension.

2.1.3. Two Dimensional Gel Electrophoresis

The purpose of using 2D gel electrophoresis is to maximally separate proteins, first by isoelectric focusing in the first dimension and then by size in the second dimension gel. In the first dimension the samples are placed in an isoelectric focusing rack filled with mineral oil and the instrument creates an electric potential along the strip. Proteins in the 1D strip move along the strip and separate according to their charge and the solution's pH. When the solution's pH is low, proteins are positively charged and move towards the negative side of the instrument; while at a high pH proteins are negatively charged and will move towards the positive side of the instrument. The proteins migrate along the pH gradient until at a specific pH their charge is zero, called the isoelectric point. At the isoelectric point the protein does not move anymore (Figure 2-

2). Thus, the proteins from a 1D strip will have a net charge of zero and will be separated. Then the 1D strip is ready for the second dimension [20].

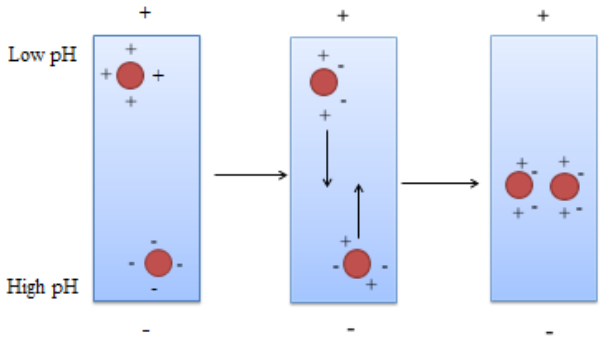


Figure 2-2. Using Isoelectric Focusing to Separate Proteins.

Following separation by isoelectric focusing, the 1D strip is placed on top of a polyacrylamide sheet and treated with sodium dodecyl sulfate (SDS) to denature the proteins and give them a negative charge. When the 2D gel electrophoresis instrument applies an electrical potential, proteins from the 1D strip will be attracted to the positive side of the gel and migrate through it. Proteins with a lighter mass will migrate through the gel more rapidly than larger proteins (Figure 2-3) [21].

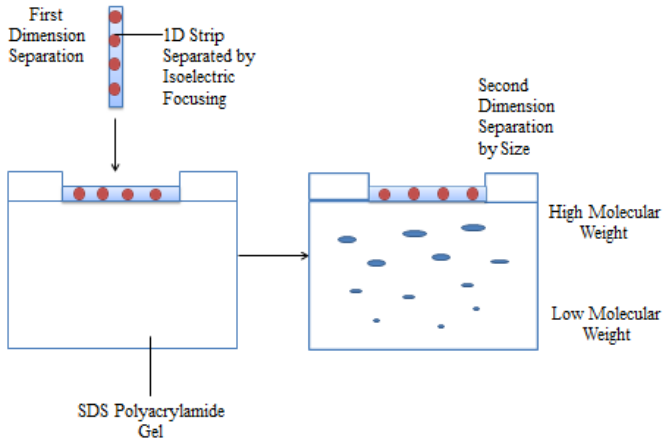


Figure 2-3. Using 2D Gel Electrophoresis to Separate Proteins.

2.1.4 Coomassie Blue Absorbance and Intensity

Once the proteins are separated they are stained with Coomassie Blue so that the amount of protein in a spot on the 2D gel can be quantified by UV absorption. The Coomassie blue interacts with the protein's amino acids primarily through the negative charges from the dye's sulfate groups being attracted to the positive side chains of the protein. Other interactions are between the aromatic rings of the protein and dye by π-electron stacking and between the hydrophobic groups of the dye and protein amino acid side chains. These interactions cause the proteins to bind to Coomassie blue molecules to make a protein-dye complex (Figure 2-4). This complex stabilizes the Coomassie dye molecules and proteins, making the spots blue and visible and shifts the absorption spectrum to a maximum of 595 nm [22].

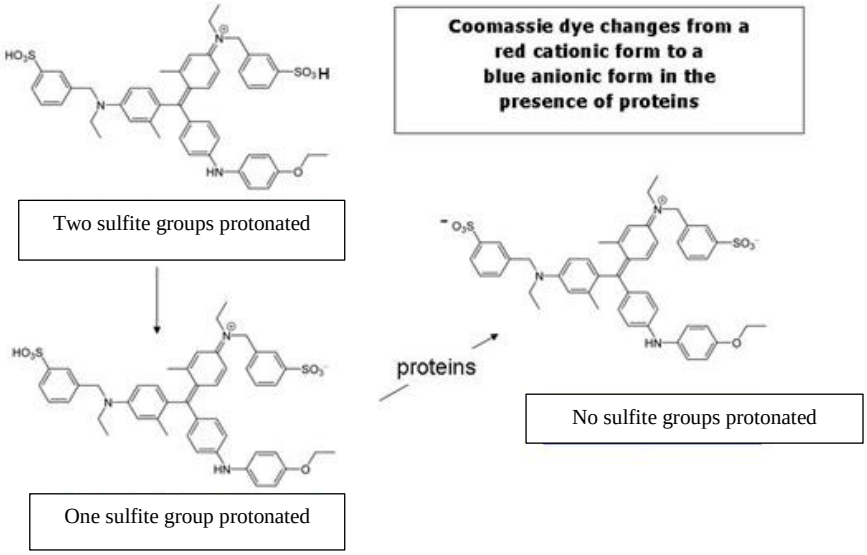


Figure 2-4. Coomassie Brilliant Blue G-250 Chemical Structures. Figure adapted from reference [22].

2.1.5 Protein Quantification by ImageMaster

The Coomassie blue stained gels were imaged by UMAX 2100 XL scanner (UMAX Technologies, Taiwan) with MagicScan software where the digital gel images were analyzed by software called ImageMaster. This software combines the measured intensity of a spot over a given area into a volume (Figure 2-5).

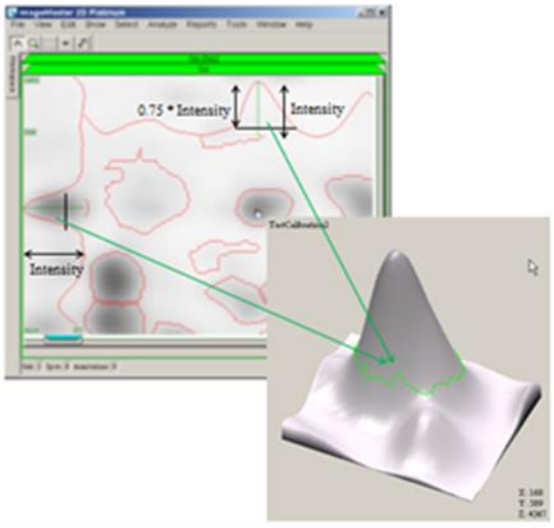


Figure 2-5. ImageMaster measuring volume. Figure adapted from reference [23].

ImageMaster also calculates %volume for each spot based upon the total volume of spots on the 2D gel. The advantage of calculating %volume is that this negates environmental error and human error (such as protein loading and staining) by normalizing the volume of an individual spot. The %volume is proportional to the concentration of the protein within the extracted protein sample [23]. The ImageMaster software automatically identified which spots on the replicate 2D gels had average %volume values that were significantly different ($p < 0.05$) than the corresponding spot on the replicate control 2D gels. Of the typically 300 spots identified on

the 2D gel images, only five spots were found to be significantly different from the controls in this study.

2.1.6. Trypsin Digestion

The purpose of trypsin digestion of excised protein spots from the 2D gel is to cleave proteins into peptides for MALDI TOF-TOF analysis. Trypsin is an enzyme that cleaves proteins at the carboxyl side of lysine and arginine, unless there is a proline following it because proline has a bulky-cyclic like side chain. Trypsin cleaves proteins by using three of its amino acids, histidine, aspartate and serine. Aspartate attracts amino acids lysine and arginine from a protein while histidine assists serine in cleaving the protein into peptides. Once the trypsin digestion is complete, the peptides from an individual spot on the 2D gel are lyophilized to make them more concentrated for MALDI TOF-TOF analysis.

2.1.7. MALDI TOF-TOF Analysis Instrumentation and Software

MALDI TOF-TOF is an instrument used to identify proteins and peptides. It works by using an Nd-YAG 200 Hz laser to ablate the matrix that the peptide sample is mixed into. The matrix absorbs light from the laser and becomes ionized in addition to the peptide sample. The matrix and peptides have to be ionized so that the instrument's electric field can pull them into the flight tube and accelerate them. Then the ions are separated in the flight tube by time-of-flight and analyzed when reaching the detector (Figure 2-6). In the first measurement, the mass of the matrix-assisted laser ablated peptides are determined by time-of-flight through the instrument (MALDI MS). In the second measurement nitrogen collision gas is then introduced into the system. During this measurement the nitrogen molecules collide with the peptides to create smaller fragments to produce a characteristic fragmentation spectrum (MALDI MS/MS) [24].

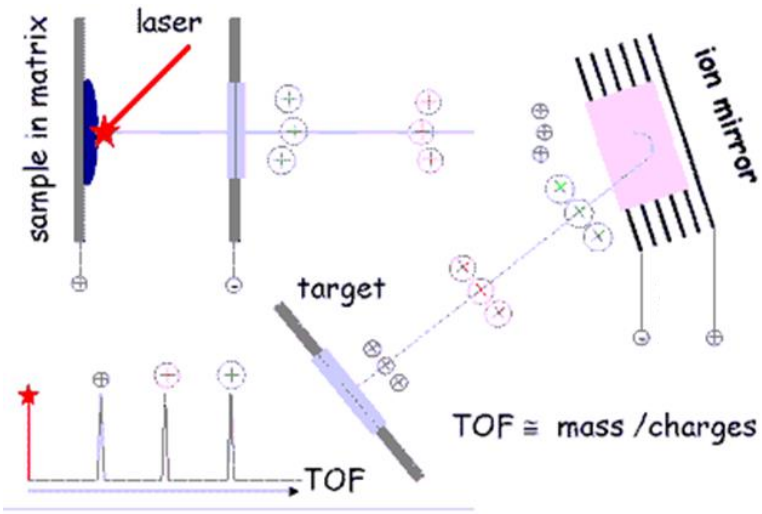


Figure 2-6. MALDI TOF Mechanism. Figure adapted from reference [25].

To make each process more effective, a reflectron mode is applied to the instrument before running MALDI TOF-TOF. The advantage of the reflectron is that the ions reach the detector at the same time to produce an m/z spectrum with pristine peaks. It works by making the heavy ions go through a shorter track in the instrument while the lighter ions go through a longer track.

When the MALDI MS/MS data is obtained it is transferred to the GPS explorer. The explorer is used because it has the Mascot search engine. The Mascot search engine is a system that uses databases, either the National Center for Biotechnology Information (NCBI) or Swissprot, to analyze mass spectrometry data to identify the peptides which produced the various peaks in the mass spectrum. The amino acid sequences from the peptide samples are matched to the sequences in the NCBI or Swissprot databases. Once there is a match, the Mascot search engine traces the amino acid sequence from the peptide back to the protein, identifying it. Both NCBI and Swissprot can identify proteins but there are some differences. NCBI takes longer to run than Swissprot because it runs all peptide sequences. Contrariwise, Swissprot is quicker to run because it edits the peptide sequences, for instance, it will not analyze a sequence that is

redundant thus there are less matching possibilities [26]. NCBI was chosen because it analyzes all peptide sequences.

The various parameters to define the search methods and conditions in the Mascot search are shown in table 2-1.

Mascot Ion Search Parameters	
Database	NCBI
Taxonomy	Mus Musculus
Enzyme	Trypsin
Fixed Modifications	Carbamidomethyl (C)
Variable Modifications	Oxidation (M)
Missed Cleavages	One
Peptide Mass Tolerances	±100 ppm
Peptide Charge	1+
Mass Values	Monoisotopic
Fragment Mass Tolerance	±0.25 Da
Report Top	10 hits

Table 2-1. Mascot Ion Search Parameters.

The rest of the sections in this chapter describe the details of each part of the experimental procedure.

2.2 Obtaining Tongue Samples for 2D Gel Electrophoresis and MALDI TOF-TOF

Unwanted adult BALB/c mice from the same breed (C5) were obtained from the University of Missouri Medical School. Mice were divided into three major groups: untreated controls, LPS injected controls, and radiated mice not previously injected with LPS. For radiation, the mice were not anesthetized; they were placed in a mouse radiation containment cage after being weighed. The control mice, were similarly weighed and then placed in the radiation containment cage, but not treated with radiation.

A linear particle accelerator (LINAC) (Siemens, Munich and Berlin, Germany) was used to give whole body external beam radiation to the mice [27]. They received total body irradiation (TBI) with a low linear energy transfer (LET) photon beam to doses of 2 Gy, 4 Gy and 7 Gy (TBI greater than 7 Gy was avoided because the LD50 at 30 days for mice is 8 Gy) [11]. The mice were observed for any loss of motor skills and for weight loss prior to sacrifice. The irradiated mice and LPS injected mice were sacrificed at 1 hour, 24 hours and 72 hours post radiation. The controls were sacrificed immediately upon removal from the radiation containment cage. All animals were sacrificed by decapitation.

The animal work was performed under the University of Missouri Animal Use and Care Protocol number 6561. Following sacrifice, tongues were removed promptly by scissors and forceps then placed in a 1 mL microcentrifuge tube which was immediately put in a container filled with liquid nitrogen. Then the samples were stored in a -80°C freezer until they were used for the rest of the experimental procedures.

2.3 Freeze Drying and Grinding Tongue Samples

The tongue samples were freeze dried promptly upon removal, by placing them inside a mortar, with liquid nitrogen and grinding by hand to a powder form.

2.4 Protein Extraction Steps

The method used to extract proteins was adapted from reference [28]. The frozen powdered tongue was placed in a protein extraction solution, consisting of 2.5 mL of tris-phenol mixture and 2.5 mL of extraction media. The mixture is phenol buffered to pH 8.8 with tris (hydroxymethyl) aminomethane (hereafter "tris") (Acros Organics, Geel, Belgium). The extraction media is composed of 0.1 M tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose (Fisher Scientific, Hampton, NH).

The extraction mixture was added to the powdered tongue sample in the mortar, and mixed using the pestle and transferred to a 14 mL microcentrifuge tube. The sample mixture was agitated for 30 minutes at 4° C then centrifuged for 30 minutes at 5000 relative centrifugal force (RCF), at 4° C. The phenol phase containing extracted proteins was removed with a 1 mL pipette and transferred to a 50 mL centrifuge tube. An additional 2.5 mL tris-phenol was added to the residual aqueous phase of the 14 mL microcentrifuge tube. This mixture was again agitated, centrifuged and the phenol phase was removed and transferred to the 50 mL centrifuge tube containing the first extraction. The combined phenol extraction solution was then washed with 10 mL of fresh tris-EDTA/sucrose extraction media. The mixture was agitated, centrifuged and the phenol phase was transferred to a new 50 mL centrifuge tube.

2.5 Protein Precipitation and Sample Dilutions

To finish precipitating the proteins and to dilute the sample for passive rehydration standard literature procedures were followed [19, 23, and 28]. The phenol-extracted proteins were precipitated by adding 10 mL of ice-cold 0.1 M ammonium acetate in 100% methanol to the phenol phase. The sample mixture was then vortexed and stored at -80°C overnight. The sample was centrifuged for 30 minutes at 5000 RCF, 4°C to create a white pellet. The methanol solution was decanted and the pellet sample was washed twice with ice-cold 0.1 M ammonium acetate in methanol containing 10 mM DTT. The mixture was then washed twice with ice-cold 80% acetone containing 10 mM DTT. The sample was centrifuged for 30 minutes in between each wash and the resulting pellet was stored in the -20°C refrigerator for 20 minutes prior to the next wash. The total protein concentration in the pellet was determined (see below) and the sample diluted with IEF buffer to 70 µg protein/µL for quantification of individual proteins. The dilution/quantification measurements were performed in triplicate. Once the triplicate dilution samples were quantified, one additional 1D and 2D separation was performed with the mouse sample that gave the best protein separation from the three triplicate analyses. In this case, a total protein concentration of 240 µg/µL was used in the one- and two-dimensional separations in order to provide enough material for MALDI MS/MS identification.

The diluted sample needed isoelectric focusing (IEF) buffer composed of 8 M urea (Fisher BioReagents, Hampton, NH), 2 M thiourea (Fisher Scientific, Hampton, NH), 4% of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, “CHAPS” (Research Products International Corporation, Mount Prospect, IL), 2% of 3-(4-Heptyl)phenyl-3-hydroxypropyl) dimethylammoniopropanesulfonate, “C7BzO” (Acros Organics, Geel, Belgium) and 100 mM

DTT (Sigma-Aldrich, St. Louis, MO). Seventy five microliters of the IEF buffer was added to the protein pellet and the mixture was vortexed for an hour.

To determine the total amount of protein sample loaded to the equilibration rack for passive rehydration and the first dimension of the 2D electrophoresis, serial dilutions were made in the ratio of 1:20, 1:40, and 1:80. These were compared to a standard, ovalbumin (Invitrogen Molecular Probes, Eugene, Oregon) in IEF buffer; the standard series concentrations were 1.25, 0.625, 0.313, 0.156, 0.078, 0.039 and 0 µg/µL. One microliter of the diluted samples and standards were pipetted to assay paper on a microarray plate (Whatman International Ltd, Little, Chalfont). The standards were analyzed in duplicate and the diluted samples were analyzed in triplicate.

The assay paper was dried, washed with methanol to remove impurities, dried again and washed with EZQ component A (Molecular Probes by Life Technologies, Eugene, Oregon) to coat the proteins with a fluorescent agent. The sheet was then washed 3 times (2 minutes each) with a solution containing 7% glacial acetic acid, 10% methanol (standard-grade), and 83% Milli-Q water to remove impurities. After the assay paper was dried, it was placed in the microarray which in turn was placed in the Fujifilm FLA 5000 to be analyzed.

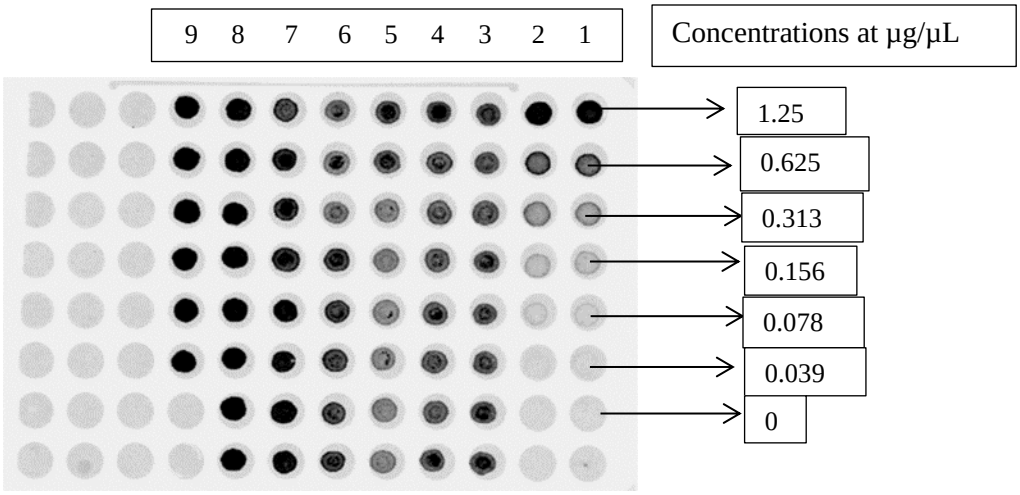


Figure 2-7. Microarray Image dilutions of standards and 24 hours post 4 Gy and 7 Gy samples.

Figure 2-7 is a microarray fluorescence image of samples and standards at a laser excitation wavelength of 473 nm. Columns 1 and 2 are the calibration standards with serial dilutions labelled. Columns 3 through 9 are samples with serial dilutions. Figure 2-8 is a standard calibration curve which is compared to the sample dilutions to determine protein concentration. The blank (0.0) is closer to the 0.039 due to background.

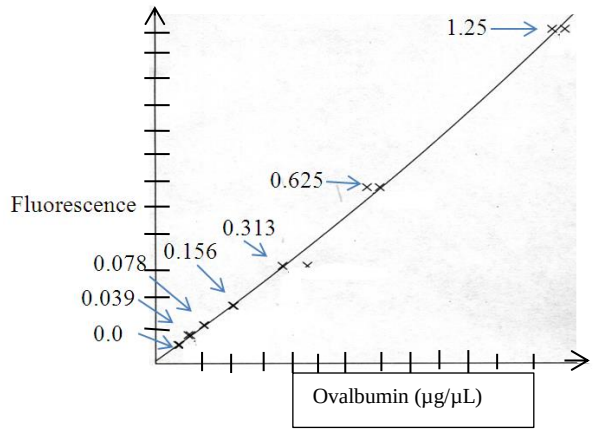


Figure 2-8. Density Calibration Curve used for 24 hours post 4 Gy and 7 Gy samples.

The sample mixture with a total protein amount of 70 µg/µL was mixed with ampholytes pH 3-10 (2.5 µL); glycerol (6.0 µL) and 2-HED (5.5 µL) and was then loaded for passive rehydration by placing a 1D strip (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) on top of the sample and filling with mineral oil to prevent dehydration. The 1D gels were made in triplicates, one gel per sample to later run 2D gel electrophoresis for quantification. Once the 2D gels were quantified a fresh new gel was run by selecting the sample from the triplicates that separated the best and had the most spots. This new 2D gel had a total protein amount of 240 µg/µL which is the appropriate concentration to excise proteins for trypsin digestion and MALDI TOF-TOF analysis.

2.6 First Dimension Gel Separation using Isoelectric Focusing

After passive rehydration, the denatured proteins in a sample were separated by their isoelectric points using isoelectric focusing using a standard procedure [29]. Isoelectric focusing was done by first removing the 1D gel strip with the sample, from the passive rehydration rack holder. It was placed on the mineral oil filled focusing (cup loading) tray, gel side up. Two electrode wicks were dipped in a beaker filled with tap water to run isoelectric focusing. Then the wicks were placed on the ends of the gel strip, and the electrodes were placed on top of the wicks.

The focusing apparatus (BIO RAD Protean IEF Cell, Hercules, CA) was set to accommodate a 13 cm long load, without rehydration. The focusing instrument was optimized for volt hours (V hrs.) which was taken from reference [30] and set up as followed; step 1 250 volts at 250 V hrs., step 2 1000 volts at 500 V hrs., step 3 8000 volts at 2 V hrs., step 4 8000 volts at 30,000 V hrs., and step 5 500 volts at 24 V hrs. The strip was run overnight.

2.7 Second Dimension Gel Separation by Mass

2.7.1 Fabrication of SDS gels

The procedure to make and run SDS gels was adapted from reference [29]. The 2D SDS gels were made with twelve percent SDS-PAGE gels (4 gels) by mixing 1.5 M tris pH 8.8, Milli-Q water (56.9 mL), acrylamide (68 mL), 10% SDS (1.7 mL), three 1 mL microcentrifuge tubes of ammonium persulfide (APS) solution (0.057 g of APS mixed with 1 mL of Milli-Q water per tube) and 0.17 mL of tetramethylethylenediamine (TEMED). This was used for one set of triplicates since the fourth gel was used as a blank. However, for two triplicate sets the volumes (and weights) were doubled to prepare a solution for 6 gels. A 12% SDS-PAGE gel was chosen because the proteins migrate with the most consistent spacing at this SDS concentration [31]. While the polyacrylamide gel mixture was prepared, gel blocks were cleaned with distilled water and soap to prevent streaking. The blocks were then wiped with ethanol, dried and set up with the rest of the apparatus. TEMED was added last because it accelerates polymerization of the acrylamide.

The gel sheets were filled with 26 mL of polyacrylamide gel mixture. A layer with 600 μL of n-butanol and 600 μL Milli-Q water was added to the top of each gel to prevent oxygen from entering the gels and inhibiting polymerization.

Once the gels were polymerized, the n-butanol/water layer was removed and the gels were rinsed with distilled water. Then a stacker was made of 2.66 mL acrylamide, 5 mL of 0.5 M tris-HCl pH 6.8 filtered, 0.2 mL 10% SDS, Milli-Q water, 0.02 g APS in 1 mL of Milli-Q water and 0.02 mL TEMED which was doubled for 6 gels. Seven milliliters of stacker was poured onto the gels. Then the stacker was layered with n-butanol and water.

While the SDS gels were being made, the isoelectric focusing apparatus with 1D sample strips was re-programmed to run for a shorter time. The short program was used to keep the proteins tightly separated. The short program's parameters were by time instead of volt hours. This 13 cm short program was; step 1 250 volts for 5 minutes, step 2 1000 volts for 5 minutes, step 3 8000 volts for 30 minutes, step 4 8000 volts for 30 minutes and step 5 500 volts for 3 hours.

The strips were removed from the isoelectric focusing rack and placed on an equilibration rack. The equilibration rack was filled with a DTT buffer solution, which was made by adding urea (9.94 g), 0.2 g of DTT, 10% SDS (5.6 mL), glycerol (5.6 mL), and 1.5 M tris pH 8.8 (2.27 mL). This was sufficient volume for 3 strips (doubled for 6 strips). The strip filled rack was placed on a rocker for 15 minutes to denature the proteins and verify protein impregnation of the strip by the appearance of a blue color (bromophenol blue in the DTT solution). The DTT was removed and the rack was filled with an iodoacetic acid (IAA) buffered solution, which also contained urea (9.94 g), 10% SDS (5.6 mL), glycerol (5.6 mL), and 1.5 M tris pH 8.8 (2.27 mL) and 0.25 g of IAA (doubled for 6 strips) which was put on a rocker for 15 minutes. IAA rehydrates the samples and prevents the reformation of disulfide bridges [32]. IAA was removed, and the rack was filled with 1.0 X SDS running buffer for 10 minutes. The 1.0 X SDS buffer was made by taking 10 X SDS buffer, (composed of 30.25 g Tris Base Ultrapure, 144.12 g Glycine, 10 g SDS and 1 L of Milli-Q water) and diluting to 1.0 X SDS buffer (10% of 10 X SDS buffer to 90% Milli-Q water) in a graduated cylinder. The 1.0 X SDS buffer was used to wash away the residual mineral oil.

The n-butanol and water layer from the 2D gel apparatus was removed and washed with distilled water. Then the apparatus was filled with 1.0 X SDS buffer and the 1D gel strips were placed on top of the apparatus. The SDS buffer was removed and a new wick was marked by a spectra

multicolor low range protein (Thermo Scientific Waltham, MA) and warm agarose gel. The 1.0 X SDS buffer was replaced with agarose gel. Additional 1.0 X SDS buffer was used to fill the gel container.

The gel apparatus was placed into the container where the rest of the 1.0 X SDS buffer was poured into the container until it reached the maximum and filled the yellow container that was attached to the gels. The electrodes were hooked to the power supply and the voltage was applied to the gel and allowed to run overnight. For one container, the instrument was set at 600 V, 14 mA and 100 W. If two containers shared the same power supply then the instrument was set at 600 V, 28 mA and 100 W. The gels were then removed from the apparatus and washed three times with Milli-Q water (10 minutes each) to remove the SDS (because it can bind to other proteins instead of the Coomassie blue molecules). Then the gels were washed with Coomassie blue and placed on a rocker overnight. To make the Coomassie blue for a 4 liter bottle, 842 mL of ethanol (20%), 75.2 mL of phosphorous acid (1.6%), 320 g of ammonium sulfate (8%) and 3.2 g of Brilliant Blue G-250 (0.08%) were mixed.

2.8 Quantification by Absorption Measurements

The quantification procedure for the proteins from the 2D gel electrophoresis was adapted from the reference [23]. The gels were stained with Brilliant Blue G-250 Coomassie (as above) then washed three times with Milli-Q water (10 minutes each). Then they were imaged by a UMAX 2100 XL scanner using the software package MagicScan. The gel images were transferred to a software package called ImageMaster that compares spots from one gel to other gels to determine if a protein spot's percent volume is different between gels.

This was done by first comparing the gels in terms of groups, e.g. 2 Gy. Each group contained triplicate gels, or three tongue samples. First, 15 spots from each gel were manually matched by placing a landmark on them. Then the software matched the rest of the spots by their intensity of the Coomassie blue, the volume and area of that spot. Next, the groups were matched. A group consisted of 3 gels in which each sample received the same treatment (e.g. three 2 Gy tongues) or non-treatment (e.g. 3 controls). After that, they were matched by class which is when one group (e.g. 2 Gy) is compared to a different group (e.g. control). This was done by choosing the gel with the most spots from each group and making it the master gel. Next, the spots from the master gel in each group, e.g. 2 Gy vs. control, were matched based on class.

Once all of the matches were done, the gel images were inspected to find and remove artifacts from the classes. As illustrated in Figure 2-9, these artifacts needed to be removed by the analyst before comparing the samples and obtaining the %volume because artifacts (most often dark particles) add to the volume which affects the %volume and concentration comparison. This was done by using the software to cut the artifacts out of the spot.

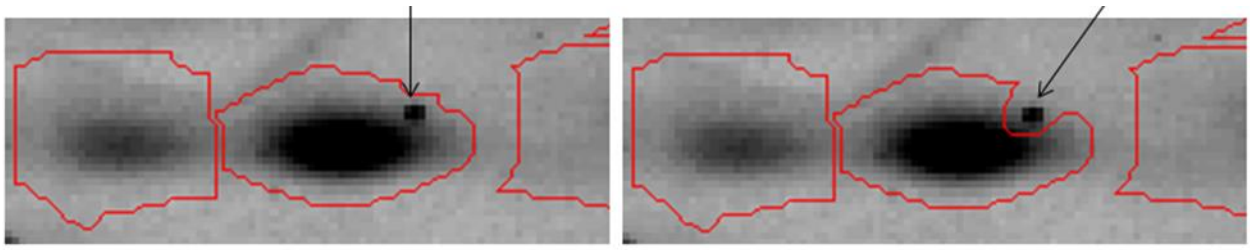


Figure 2-9. Spot with artifact (left) and spot without artifact (right) from a 72.5 µg BCA spike in ImageMaster.

The relative amount of protein in each sample was taken from artifact corrected %volume values.

2.9 Trypsin Digestion

The trypsin digestion procedure was taken from references [33 and 34]. A 1.5 mm gel excision tool was used to excise spots that were subjected to trypsin digestion. To perform the trypsin digestion, these gel plugs were placed in 1.5 mL microcentrifuge tubes and individually washed with 500 µL of wash solution (250 µL acetonitrile (ACN) and 250 µL of 100 mM ammonium bicarbonate) to remove the Coomassie dye. The spot sample was then vortexed for 15 minutes and the washing process was repeated three more times (centrifuged for 30 seconds in between). Then the wash solution was replaced by 500 µL of 100% ACN for dehydration. This was repeated a second time and vortexed for 20 minutes and then centrifuged (Fisher Scientific, Hampton, NH) for 10 to 20 seconds. The ACN was removed and the gel plugs air-dried for 5 minutes. During the drying period, protease digestion solution was prepared. Trypsin stock solution was made by adding 20 µL of trypsin resuspension buffer (Promega, Fitchburg, WI) to 20 µg of trypsin (Promega, Fitchburg, WI) and making 2 µL aliquots. The stock solution was diluted to 1 µg/50 µL with 50 mM ammonium bicarbonate. The gels were rehydrated with 20 µL of trypsin protease digestion solution, and placed on ice for 1 hour. After 1 hour, the trypsin solution was removed and replaced with 30 µL of 50 mM ambic to keep the gel plugs hydrated during overnight digestion (at 37° C). The samples were centrifuged 10 to 20 seconds and the supernatant (containing tryptic digest peptides) was transferred to a clean 0.5 mL microcentrifuge tube and placed on ice. The top of the tube was punctured with an 18 gauge needle to allow easy lyophilization.

Twenty five microliters of extraction solution (60 % ACN, 1 % TFA) was added to the gel plugs, vortexed, and centrifuged for 10 to 20 seconds. TFA was purchased from Honeywell Burdick & Jackson (Morristown, NJ). The supernatant (containing additional tryptic peptides) was

transferred to the same 0.5 mL microcentrifuge tube. This entire procedure was repeated and the samples flash frozen and lyophilized. After the samples were lyophilized to dryness, 3 to 5 µL of resuspension solution (50 % ACN, 0.1 % TFA) was added to each tube and vortexed. The samples were centrifuged for 10 seconds.

A mixture of 50-pmol/µL 4700 Proteomics Analyzer Calibration Mixture (des-Arg1-Bradykinin, Angiotensin, Glu1-Fibrinopeptide B and ACTH) and CHCA matrix (Fluka, St. Louis, MO) (1.0 µL-10 mg/mL in 50 % ACN, 0.1 % TFA) was pipetted onto a MALDI plate at 6 specific wells, each 0.5 µL. The supernatant (0.5 µL) was pipetted onto the same plate followed by 0.5 µL of CHCA (10 mg/mL in 50 % ACN, 0.1 % TFA). The spots were dried under a hood and the plate was loaded into the Applied Biosystems 4700 MALDI TOF for analysis.

2.10 Protein Identification by MALDI TOF-TOF Analysis

2.10.1 MALDI TOF-TOF Instrumentation

The MALDI TOF-TOF instrument settings used in this study were adapted from reference [35]. The Applied Biosystems 4700 MALDI TOF obtained spectra by setting the acquisition and processing parameters with MS and MS/MS to calibrate and run samples. For calibrating under MS, the acquisition was positive reflector MS with a mass range of 700-4000 Da, a total shots per spectrum at 2,000 and a laser intensity of 3,800. The processing method was an internal calibration MS with peak detections of 1,000 and 3,600 Da with a resolution of 12,000. For calibrating under MS/MS the acquisition method was 1kV positive Glu1-Fibrinopeptide B with total shots per spectrum at 3,500 with a precursor mass of 1570.677 Da and laser intensity of 5,000. The processing method was an internal calibration Glu1-Fibrinopeptide B with a peak detection at 500 Da (resolution 6,000), 1,000 Da (resolution 1,000) and 1,300 Da (resolution

12,000). After the calibration runs were completed, the parameters for the samples were set. The acquisition method was positive reflector MS with a mass range of 700-4000 Da, total shots per spectrum at 2,000 but a laser intensity of 4,000. The processing method was an MS Trypsin with peak detections of 900 Da (resolution 10,000), 1,300 Da (resolution 15,000) and 2,200 Da (resolution 20,000). The samples were run overnight due to the amount of samples and sent to the GPS system which had the Mascot search engine.

2.10.2 Software used for Protein Identification

The Mascot Ion Search parameters were outlined in section 2.1.7 where the data was analyzed using the NCBI database. The protein identified from each sample that had the highest histogram score and was above the significance threshold was chosen. The identified proteins are discussed in the results chapter.

2.11 Standardization

To validate the protein quantification and identification, a standard response curve was constructed for comparison. The standard chosen was bovine carbonic anhydrase (BCA) because it was closest to malate dehydrogenase in molecular weight and isoelectric point. Malate dehydrogenase was originally thought of as a potential biomarker for ionizing radiation exposure. To perform this standardization, twelve BALB/c mice that were not treated were sacrificed by decapitation. Their tongues were harvested and put in liquid nitrogen. The average weight of the tongues was 120 mg. Then 290 mg of BCA (Sigma-Aldrich, St. Louis MO) was placed in a 14 mL microcentrifuge tube. It was filled with the 0.1 M tris-HCl pH 8.8, 10 mM EDTA, 0.9 M sucrose buffer to 10 mL and vortexed. Aliquots of 1 mL were divided and stored at the -80^{0}C.

The BCA solution was added to nine of the twelve microcentrifuge tubes that had the same extraction solution from the sample processing procedure described above. Ten microliters (equivalent of 290 µg) of BCA solution was added to one set of tubes (a triplicate), 5 µL (145 µg) to another set and 2.5 µL (72.5 µg) to a third while the last set was controls with no BCA. The BCA tris-phenol sucrose mixture was vortexed. Then the rest of the sample processing procedure, isoelectric focusing, 2D gel electrophoresis, washing with Milli-Q water, Coomassie blue staining, quantification from ImageMaster, trypsin digestion and MALDI TOF-TOF identification from the irradiated tongue samples was done for the BCA spiked tongue samples.

The only difference is that for the protein dilution step, a total sample protein of 240 µg/µL was used for each gel because it was already known that the BCA spot was going to be excised whereas in the case of irradiated tongue samples, it was not known which spots would be cut out until quantification from measuring absorptions was known. In other words, the triplicate gels from the control BCA standard experiment (total sample protein loaded was 240 µg/µL) were scanned for quantification then their spots excised (and put in the -80°C freezer) for trypsin digestion and MALDI TOF-TOF identification. However, the triplicates from the irradiated tongue samples needed to be scanned for quantification and to know which spots would be excised (total sample protein loaded was 70 µg/µL). When that was done, then another gel was made for one tongue sample (total sample protein loaded was 240 µg/µL) from the triplicates to excise the spots for trypsin digestion and MALDI TOF-TOF identification.

This controlled BCA spiked experiment was successfully repeated such that BCA was identified quantified, and percent volume obtained. This produced graphs of average %volume vs. BCA amount which displayed a pattern of decreasing %volume (compared to the controls) by

decreasing BCA amount. These graphs were converted to a standard response curve that was compared to the average %volume vs. dose graphs to validate the proteins as biomarkers.

CHAPTER 3

RESULTS

3.1 2D Gels and Mascot Identification of BCA Spikes

Control experiments using BCA as a spike to the tongue samples were performed to confirm that the absorption measurements provided a signal that was proportional to the protein concentration in the tongue samples and to test that the MALDI MS/MS measurements correctly identified a known protein. BCA was used because the isoelectric point (pI) is 6.0 with a molecular weight of 31,000 Da [36] which is close to the initial potential biomarker malate dehydrogenase (pI of 4.8 and molecular weight of 35,000 Da) [37]. BCA falls in the molecular weight range of the five protein biomarkers that were identified in this project. Those protein biomarkers were 1) troponin I, skeletal, fast 2 (20,954 Da), 2) ATP synthase subunit beta, mitochondrial precursor (56,265 Da), 3) pyruvate kinase isozymes M1/M2 (58,378 Da), 4) aconitate hydratase, mitochondrial precursor (86,151 Da) and 5) alpha-actinin-2 (104,339 Da). Once the BCA gels were made, they were compared to the controls for both protein quantification and identification.

To identify BCA from the spiked samples and the controls, the BCA spot was excised in the majority of the gels, treated with trypsin digestion and studied with MALDI MS/MS. The BCA spot was located on each gel based on BCA's isoelectric point and the molecular weight. The BCA spikes were 290 µg, 145 µg and 72.5 µg. The BCA spike sample preparation, protein separation, and quantification were performed twice in triplicate. Because of the time and expense for the MALDI MS/MS, the protein identification of BCA for the spike experiments was performed on select samples from each of the two experiments.

The MALDI MS/MS data from the excised BCA spot on each gel was analyzed by the Mascot search engine which identified the protein as BCA. In Mascot, BCA is listed as carbonic anhydrase 2 (CA). All of the Mascot results for the samples are shown in Table 3-1 with the protein name carbonic anhydrase 2, the scores and percent protein sequence coverage.

Bovine Carbonic Anhydrase (BCA) Experiment 1	Protein Name	Top Scores (range)	Percent Protein Sequence Coverage
290 µg BCA (A)	Carbonic Anhydrase 2	80	63%
290 µg BCA (C)	Carbonic Anhydrase 2	89	65%
145 µg BCA (A)	Carbonic Anhydrase 2	72	52%
145 µg BCA (C)	Carbonic Anhydrase 2	80-93	57%
72.5 µg BCA (A)	Carbonic Anhydrase 2	74	60%
72.5 µg BCA (B)	Carbonic Anhydrase 2	74-86	68%
72.5 µg BCA (C)	Carbonic Anhydrase 2	100-127	64%
Bovine Carbonic Anhydrase (BCA) Experiment 2	Protein Name	Top Scores (range)	Percent Protein Sequence Coverage
Control (A)	Carbonic Anhydrase 2	74-86	64%
Control (B)	Carbonic Anhydrase 2	77-90	61%

Table 3-1. List of BCA Mascot Results with Scores and Percent Coverage.

For the majority of the gels that were analyzed by MALDI MS/MS, the percent protein sequence coverage ranged from the lower fifties to the upper sixties in the Mascot search results indicating that the peptide chains from the samples were correctly identified. Also, the protein scores were above the threshold indicating they were significant.

3.2 Converting Quantified Concentration Measurements to a Standard Response Curve

The Mascot search results identified BCA but the gels had to be quantified in order to make the standard response curve. The gels were quantified by the BCA absorption measurements and analyzed by ImageMaster to obtain the percent volume. The variation in the percent volume as a function of BCA spike is shown for the two control experiments in Figure 3-1. Also present in the bar graphs are the standard deviation as error bars (Figure 3-1).

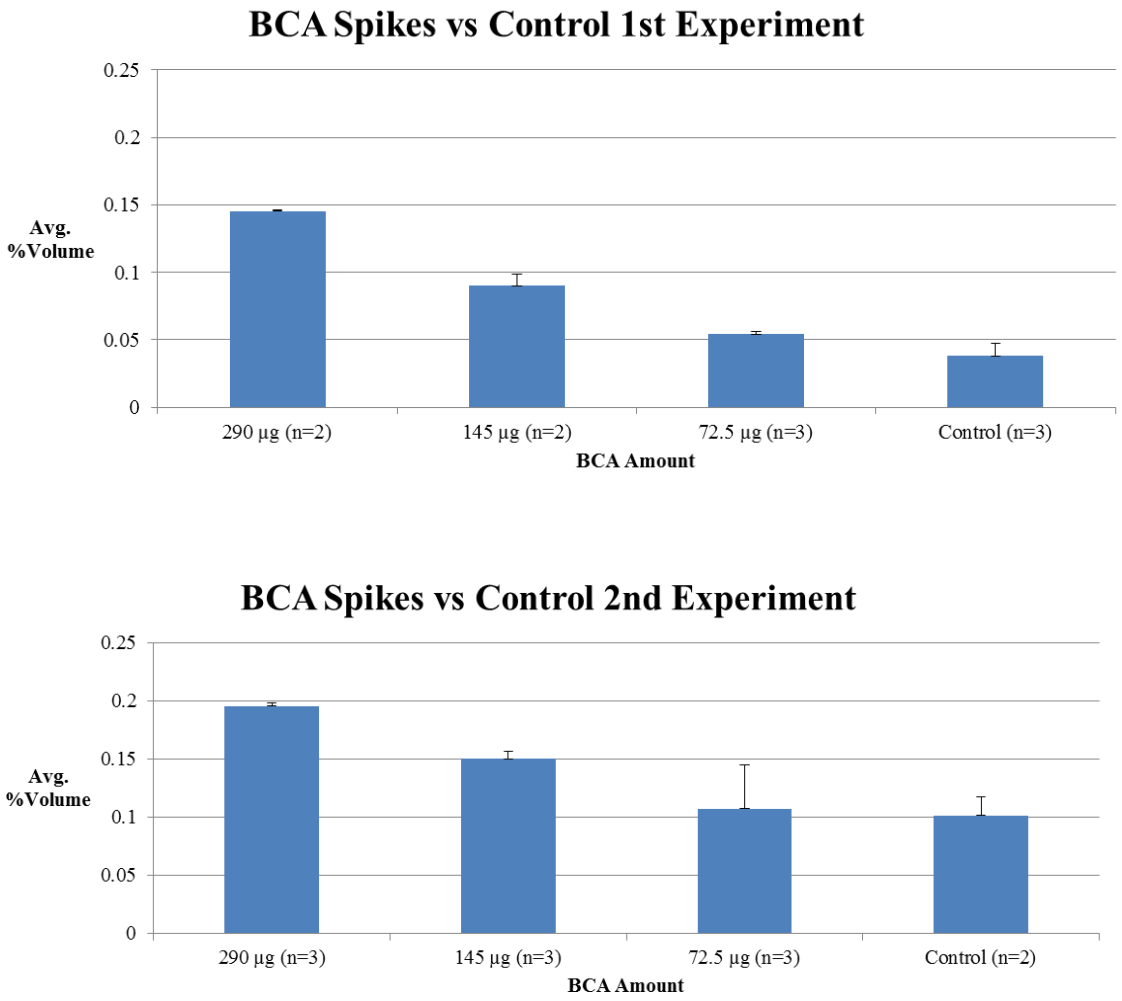


Figure 3-1. Graphs of BCA spiked samples from experiments one (upper) and two (lower).

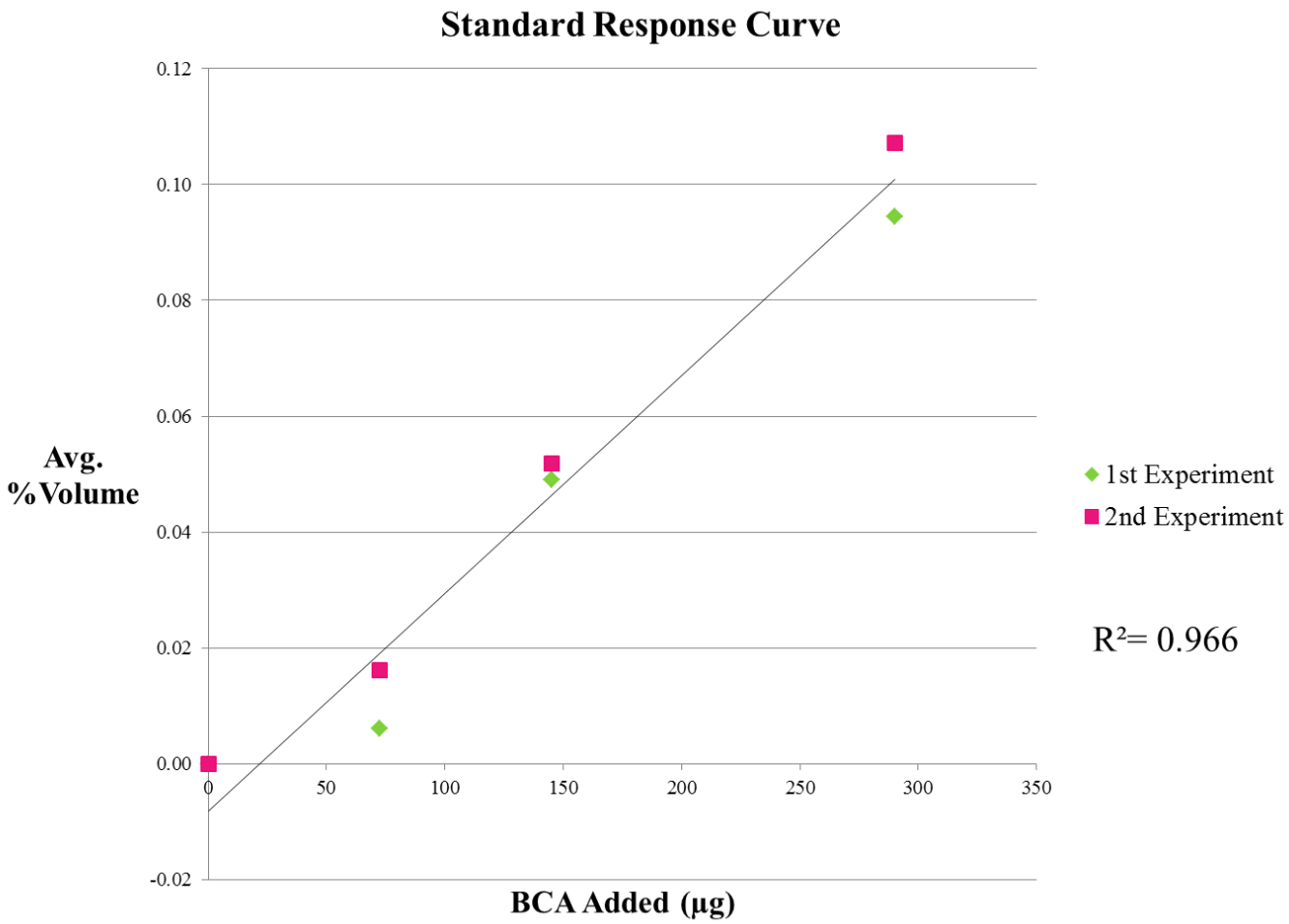


Figure 3-2. BCA Standard Response Curve.

A standard responsive curve (Figure 3-2) was constructed by subtracting the average percent volume of the control from each of the average percent volumes of the BCA spike amounts. The variation in blank subtracted %volume with BCA spike is shown in Figure 3-2 for the two control experiments. A linear fit of the average %volume from the two experiments yields an R^2 value of 0.966.

3.3 2D Gels and MALDI MS/MS Identification for IR Protein Biomarkers

When mice were exposed to TBI at 2 Gy, 4 Gy and 7 Gy and their tongue samples were compared to the controls; five biomarkers were identified in each gel based on their quantification by absorption which showed statistically significant changes when compared to the controls ($p < 0.05$). These significant changes were seen at sacrificed time intervals of 1 hour, 24 hours and 72 hours. They were troponin I, skeletal, fast 2 (spot 713), aconitate hydratase, mitochondrial precursor (spot 249), alpha-actinin-2 (spot 211), pyruvate kinase isozymes M1/M2 (spot 354) and ATP synthase subunit beta, mitochondrial precursor (spot 408). Figure 3-3 shows all of the biomarkers.

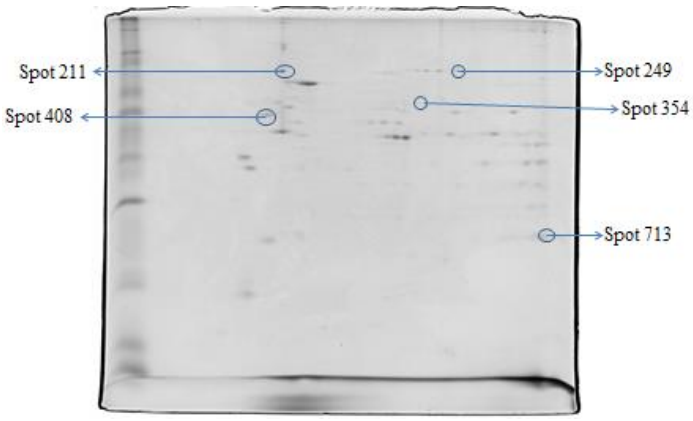
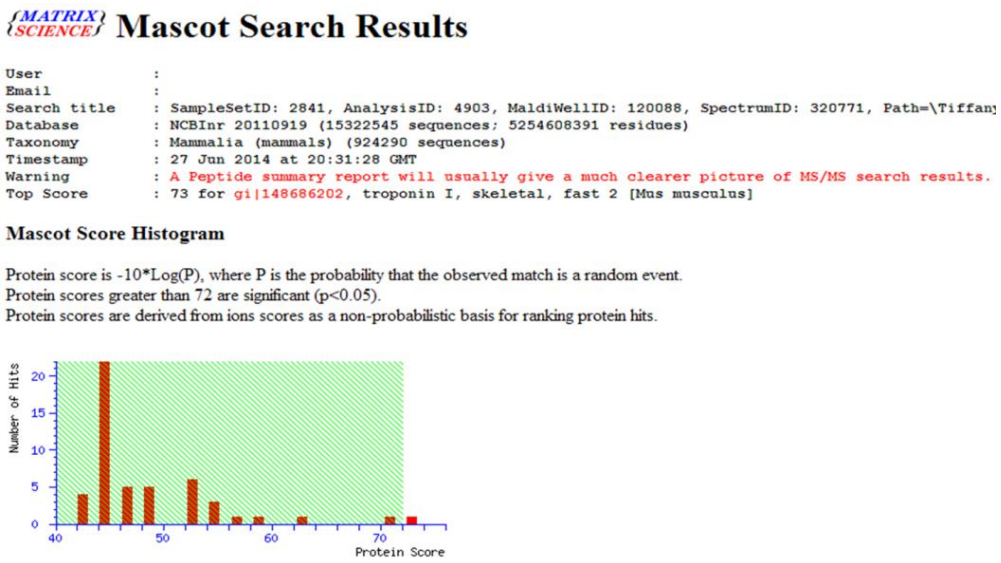


Figure 3-3. Gel of 24 hours post 4 Gy with Protein Biomarkers and Spot ID.

These spots were excised from the gels and the proteins were identified by MALDI MS/MS following trypsin digestion of the plug. The MALDI MS/MS data was transferred to the Mascot database for protein identification. The Mascot database took the peptide chain from a tongue sample and matched it to a peptide chain in the search engine. Once there was a match, Mascot traced the matched peptide to a protein for the identification. The following figures show the Mascot results of one of the protein biomarkers identified as troponin I, skeletal fast 2.

Figure 3-4 is a histogram, shown as a bar graph that displays the top score of a protein and shows what proteins were significant. A protein score above 72 passes the threshold and is thus significant because the score indicates that it was not a random match between the database and the peptide sample but instead a legitimate match based on their amino acid sequence [38]. However, anything below 72 which is in the shaded region is a random match and is not considered significant. In this example, the only protein that is above the threshold is troponin I, skeletal, fast 2 [Mus musculus] with a top score of 73.



MATRIX SCIENCE **Mascot Search Results**

User :
Email :
Search title : SampleSetID: 2841, AnalysisID: 4903, MaldiWellID: 120088, SpectrumID: 320771, Path=\Tiffany
Database : NCBInr 20110919 (15322545 sequences; 5254608391 residues)
Taxonomy : Mammalia (mammals) (924290 sequences)
Timestamp : 27 Jun 2014 at 20:31:28 GMT
Warning : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score : 73 for gi|148686202, troponin I, skeletal, fast 2 [Mus musculus]

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 72 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Figure 3-4. Mascot Search Histogram.

Below the histogram is the Index (Figure 3-5) that displays the top ten protein scores with their identification, scores, and molecular weights.

Index

	Accession	Mass	Score	Description
1.	gi|148686202	20954	73	troponin I, skeletal, fast 2 [Mus musculus]
2.	gi|6678391	21515	70	troponin I, fast skeletal muscle [Mus musculus]
3.	gi|149693696	22348	62	PREDICTED: peroxiredoxin-1-like [Equus caballus]
4.	gi|148673403	46023	58	G-rich RNA sequence binding factor 1, isoform CRA_b [Mus musculus]
5.	gi|123242975	15896	56	troponin I, skeletal, fast 2 [Mus musculus]
6.	gi|123242973	22414	55	troponin I, skeletal, fast 2 [Mus musculus]
7.	gi|55824562	21081	54	peroxiredoxin 1 [Macaca fascicularis]
8.	gi|123230136	19086	54	peroxiredoxin 1 [Mus musculus]
9.	gi|197101793	51507	54	peptidyl-prolyl cis-trans isomerase FKBP5 [Pongo abelii]
10.	gi|66773956	22423	53	RecName: Full=Peroxiredoxin-1

Figure 3-5. Protein Descriptions, Scores and Masses.

The protein to investigate is troponin I, skeletal, fast 2 [Mus musculus] since it was the only one that passed the threshold and had the highest score. The protein sequence coverage (Figure 3-6) shows how effective the Mascot search engine was in matching the peptide sequence from the sample to the database. In this example, the coverage was 54 percent.

However, not all off the peptide chains from this sample matched to the whole peptide chain in the database. This is because the 4700 MALDI TOF-TOF instrument uses nitrogen as a collision gas to collide with the peptide chains which creates smaller fragments to be analyzed in Mascot. Specifically, nitrogen breaks the carbonyl amide group bond during the collision which produces b and y ions. The way that the peptide chains were fragmented can affect matching between the sample and Mascot [39, 40].

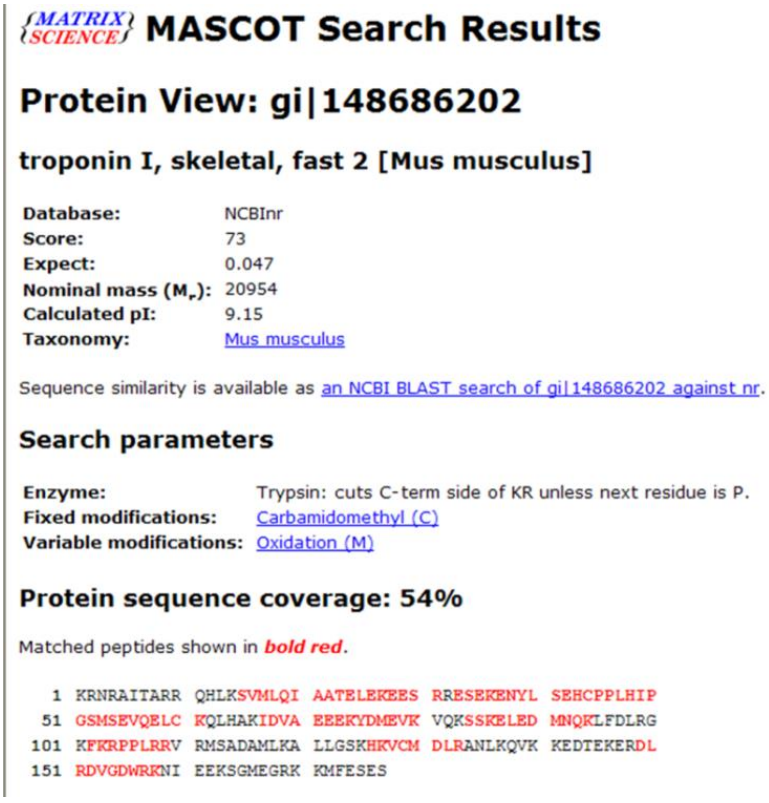


Figure 3-6. Protein View with Protein Sequence Coverage.

The key results of the Mascot search are the Mascot score histogram, the identification of that protein and the sequence coverage. The histogram displays which proteins are significant, the Index shows the scores, masses and identifications and the Protein View presents the protein sequence coverage and how the peptide chain sample matches the database. The proteins identified as up- or down-regulated in this work are shown in Table 3-2 with their names, scores and protein sequence coverage. On average, the percent protein sequence coverage was in the mid to high fifties with the exception of pyruvate kinase isozymes M1/M2 which matched at a lower percentage of 30.

Ionizing Radiation Protein Biomarkers		
Protein Name	Top Scores (Range)	Percent Protein Sequence Coverage
Troponin I, Skeletal Fast 2	73	54%
Aconitate Hydratase	300-450	56%
Alpha-Actinin-2	74-455	57%
Pyruvate Kinase Isozymes M1/M2	202-276	30%
ATP Synthase Subunit Beta, Mitochondrial Precursor	696-794	58%

Table 3-2. List of Protein Biomarkers with Scores and Percent Coverage.

3.4 Quantification Used to Determine Protein Up- and Down-Regulation for IR Biomarkers

The %volume of the IR protein biomarkers obtained from absorption measurements was compared to the control and LPS mice samples. Once the volumes were obtained, the protein biomarkers were determined to be either up-regulated or down-regulated. The quantification process was done the same way as the BCA spike experiment where ImageMaster ran the Student's *t*-test and displayed the %volume of a spot. Once the protein spot's %volume was obtained from each gel, graphs were constructed of average %volume vs. dose (compared to the controls and LPS) for different times of sacrifice post irradiation exposure. These graphs displayed either increasing or decreasing average percent protein volume in correlation to the

times when the mice were sacrificed. The standard deviations are presented as error bars. The only percent volume not shown was 2 Gy at 24 hours because the gels needed to be redone. The first graphs shown are for troponin I, skeletal fast 2 (Figure 3-7a to Figure 3-7c). In addition to the graphs, the protein biomarker's Student's *t*-test values are shown as tables (Table 3-3). The values are displayed as comparing LPS to controls, and controls to IR protein biomarkers (dependent on the times of sacrifice) when running the Student's *t*-test.

The Student's *t*-test values are based upon the 95% confidence level. To determine the 95% confidence level the degrees of freedom were calculated by v = n-1 as n being the number of samples and v the degrees of freedom. Six samples were compared (e.g. three control samples versus three 2 Gy samples) per Student's *t*-test. This gives five degrees of freedom. From there, in the student t-distribution table under two sided Student's *t*-test 95% confidence is the standard 2.571 value. After running the Student's *t*-test a list of student t-distribution values are presented for all of the matched spots in the gels. If the value is above 2.571 then the results from ImageMaster are 95% confident that there is a difference between a protein spot from the two sets of triplicates. The 95% confidence level is the equivalent to stating there is a 5% probability that the protein spot is the same in both triplicates. In this project, the controls were compared to the LPS samples and the controls were compared to the IR samples. But the student t-distribution values for comparing LPS samples to IR samples were not shown because the LPS samples were going to be compared to samples from IR mice injected with LPS which are not presently available. However, if the student t-distribution values are below 2.571 then the difference is below the 95% confidence level and the protein spot is not significant; that is below 2.571 there is no difference between that spot when comparing the controls to LPS and to the IR samples.

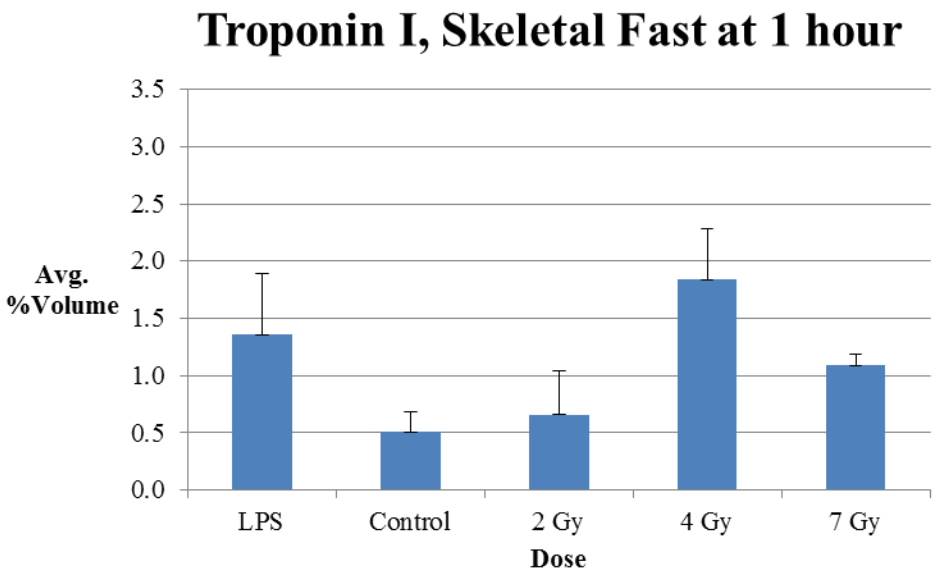


Figure 3-7a. The Average %Volume of Troponin I, Skeletal Fast 2 as a function of radiation dose in comparison to control at 1 hour.

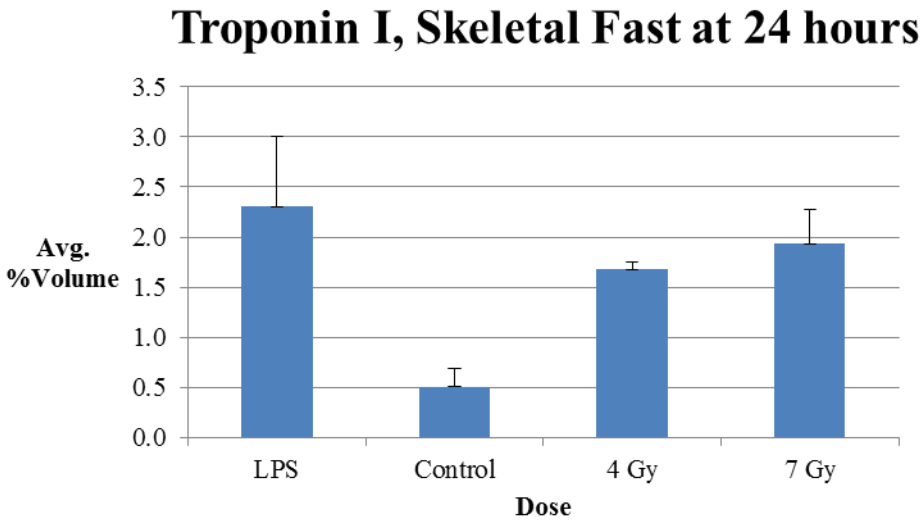


Figure 3-7b. The Average %Volume of Troponin I, Skeletal Fast 2 as a function of radiation dose in comparison to control at 24 hours.

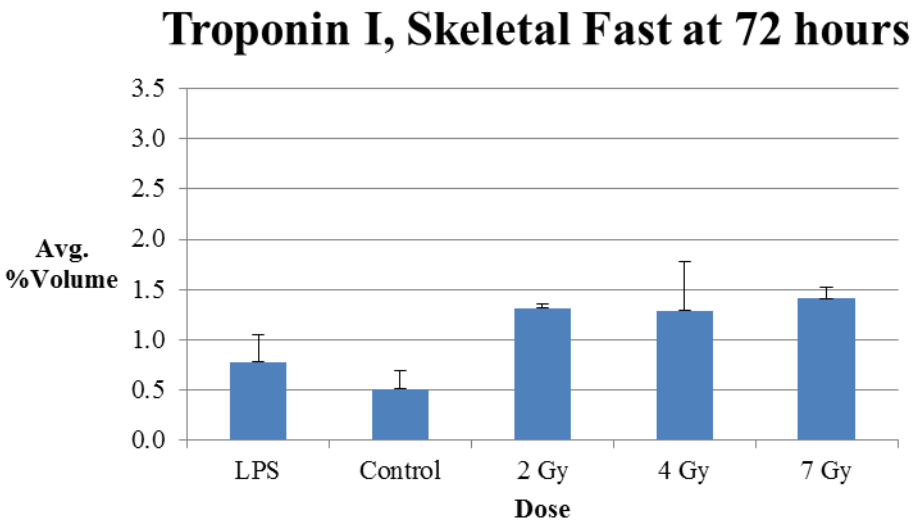


Figure 3-7c. The Average %Volume of Troponin I, Skeletal Fast 2 as a function of radiation dose in comparison to control at 72 hours.

Troponin I, skeletal, fast 2 [Mus musculus]			
Student's *t* -test values	1 hour	24 hours	72 hours
Control vs. LPS	2.74	4.33	1.42
Control vs. 2 Gy	1.03		0.83
Control vs. 4 Gy	4.56	10.63	2.61
Control vs. 7 Gy	3.55	6.36	7.59

Table 3-3. Troponin I, Skeletal Fast 2 Student's *t*-test distribution values.

Troponin I skeletal fast 2 was determined to be an up-regulated protein biomarker because in all three time points (1 hour, 24 hours and 72 hours) the radiated tongue samples at 2 Gy, 4 Gy and 7 Gy had a higher average percent volume than the controls. The increased value of troponin I was significantly different from the control at all-time points for 4 Gy and 7 Gy. Though larger, the increased value at 2 Gy was not significantly different than control. Also, the average percent volume for LPS was higher than the controls for all three time points (significantly different at 1 hour and 24 hours). This indicates that LPS caused an inflammatory response that led to an up-

regulation in the protein. Troponin I skeletal fast 2 was the only protein identified in this work that had an up-regulated response to ionizing radiation exposure; the rest of the identified biomarkers were down-regulated.

One of the protein biomarkers that were down-regulated was aconitate hydratase, mitochondrial precursor according to the average % volume (Figure 3-8a to Figure 3-8c) and the Student's *t*-test values (Table 3-4).

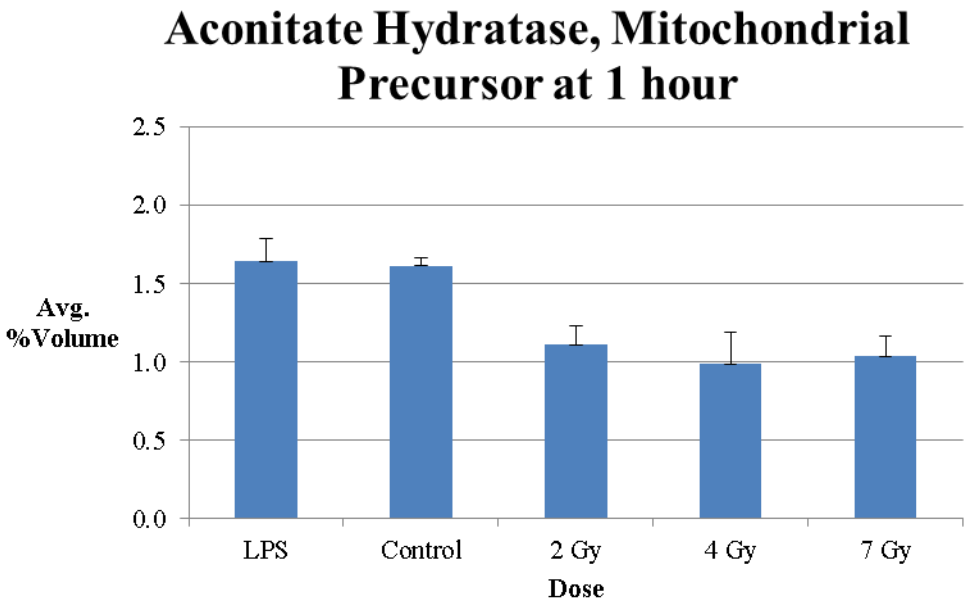


Figure 3-8a. The Average %Volume of Aconitate Hydratase, Mitochondrial Precursor as a function of radiation dose in comparison to control at 1 hour.

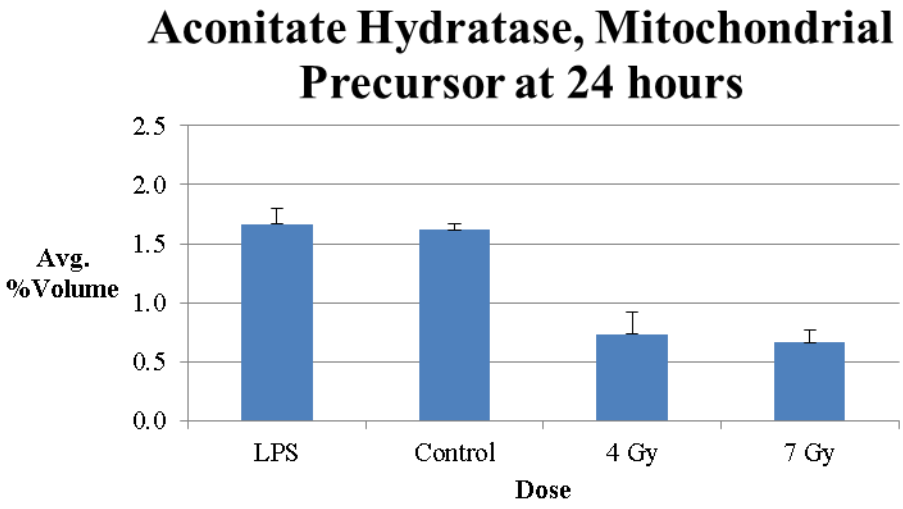


Figure 3-8b. The Average %Volume of Aconitate Hydratase, Mitochondrial Precursor as a function of radiation dose in comparison to control at 24 hours.

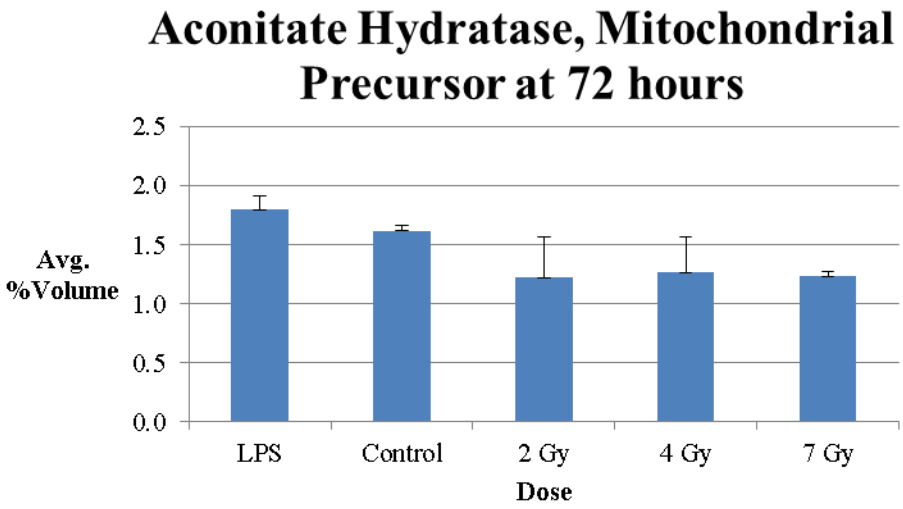


Figure 3-8c. The Average %Volume of Aconitate Hydratase, Mitochondrial Precursor as a function of radiation dose in comparison to control at 72 hours.

Aconitate hydratase, mitochondrial precursor [Mus musculus]			
Student's *t* -test values	1 hour	24 hours	72 hours
Control vs. LPS	0.37	0.65	2.47
Control vs. 2 Gy	6.79		1.92
Control vs. 4 Gy	5.11	7.79	1.97
Control vs. 7 Gy	7.17	13.21	10.2

Table 3-4. Aconitate Hydratase, Mitochondrial Precursor Student's *t*-test distribution values.

Aconitate hydratase, mitochondrial precursor is a down-regulated protein biomarker because in all three graphs at 1 hour, 24 hours and 72 hours the radiated samples were consistently lower in average %volume compared to the controls and to the LPS samples. In addition, the Student's *t*-test values display a significant protein change at 2 Gy, 4 Gy and 7 Gy at 1 hour and 24 hours. The average %volume and the Student's *t*-test values of the LPS samples is not significantly different than the controls at all three time points. The most drastic decrease in average %volume from the IR protein biomarkers is at 24 hours which was below 1.0 percent while at 1 hour and 72 hours the average percent volumes of 2 Gy, 4 Gy and 7 Gy were above 1.

The next down-regulated protein biomarker was alpha-actinin-2 according to the average % volume (Figure 3-9a to Figure 3-9c) and Student's *t*-test values (Table 3-5).

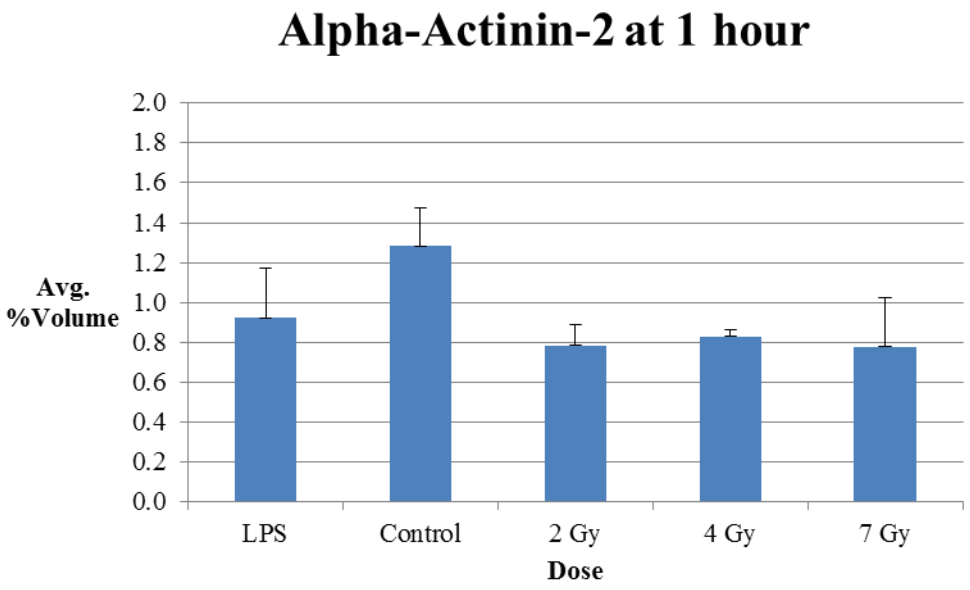


Figure 3-9a. The Average %Volume of Alpha-Actinin-2 as a function of radiation dose in comparison to control at 1 hour.

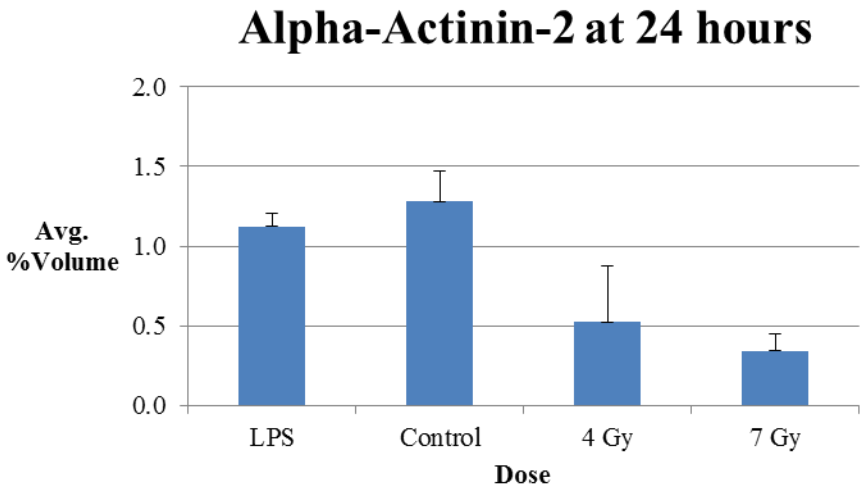


Figure 3-9b. The Average %Volume of Alpha-Actinin-2 as a function of radiation dose in comparison to control at 24 hours.

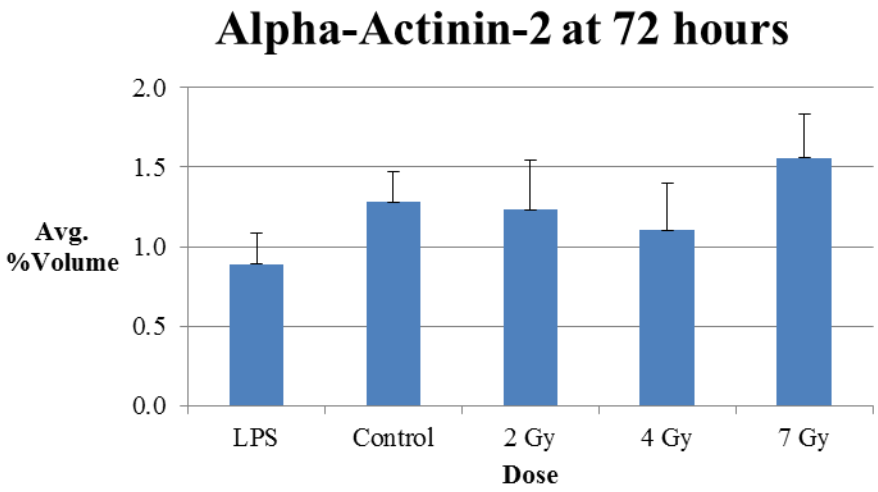


Figure 3-9c. The Average %Volume of Alpha-Actinin-2 as a function of radiation dose in comparison to control at 72 hours.

Alpha-actinin-2 [Mus musculus]			
Student's *t* -test values	1 hour	24 hours	72 hours
Control vs. LPS	1.95	1.27	2.48
Control vs. 2 Gy	3.93		0.22
Control vs. 4 Gy	4.02	2.89	0.85
Control vs. 7 Gy	2.79	6.1	1.47

Table 3-5. Alpha-Actinin-2 Student's *t*-test distribution values.

Alpha-actinin-2 is a down-regulated protein biomarker because in the 1 hour and 24 hours graphs, the average %volumes of 2 Gy, 4 Gy and 7 Gy doses were lower than the controls and LPS samples. Also, the Student's *t*-test values for 2 Gy, 4 Gy and 7 Gy were higher than 2.571 at 1 hour and 24 hours which showed a significant change. The most dramatic change caused by ionizing radiation exposure for this protein is at 24 hours because that is the largest decrease in average percent volume for the 2 Gy, 4 Gy and 7 Gy samples. Although not statistically significant, LPS consistently has a lower %volume in comparison to the controls.

After alpha-actinin-2, was the down-regulated protein biomarker pyruvate kinase isozymes M1/M2 according to the average %volume (Figure 3-10a to Figure 3-10c) and the Student's *t*-test values (Table 3-6).

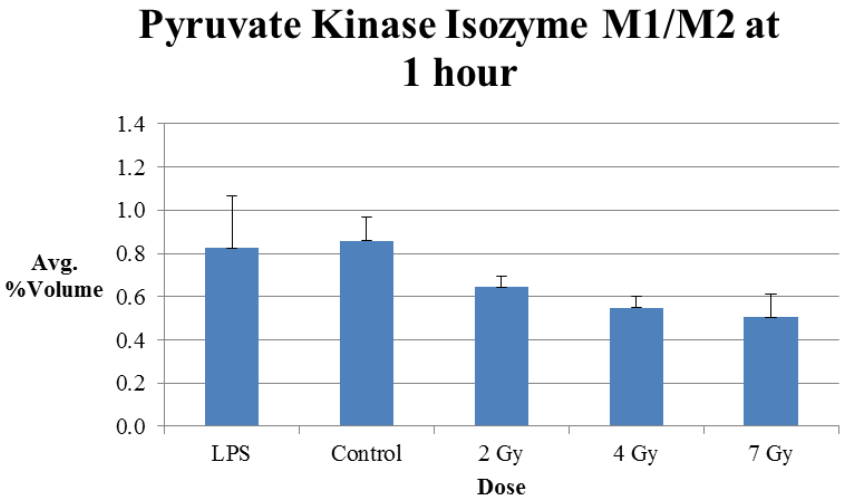


Figure 3-10a. The Average %Volume of Pyruvate Kinase Isozymes M1/M2 as a function of radiation dose in comparison to control at 1 hour.

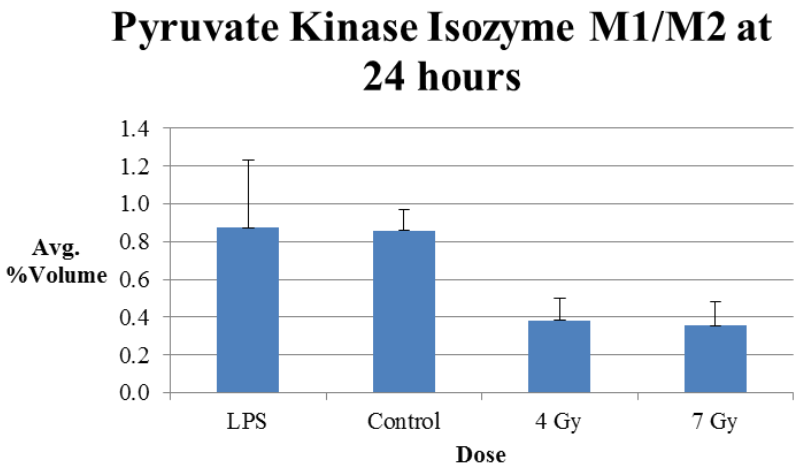


Figure 3-10b. The Average %Volume of Pyruvate Kinase Isozymes M1/M2 as a function of radiation dose in comparison to control at 24 hours.

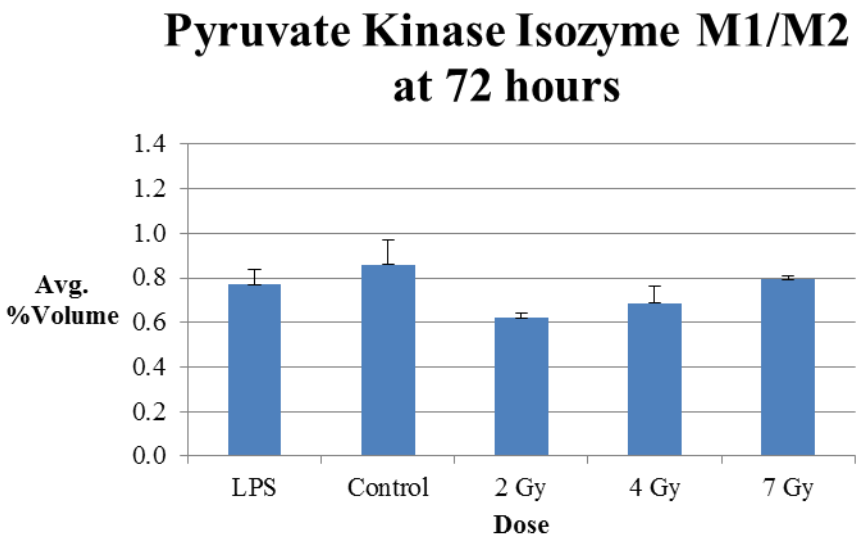


Figure 3-10c. The Average %Volume of Pyruvate Kinase Isozymes M1/M2 as a function of radiation dose in comparison to control at 72 hours.

Pyruvate kinase isozymes M1/M2 [Mus musculus]			
Student's *t* -test values	1 hour	24 hours	72 hours
Control vs. LPS	0.19	0.08	1.12
Control vs. 2 Gy	3.02		2.04
Control vs. 4 Gy	4.31	5.02	2.14
Control vs. 7 Gy	3.94	3.67	0.95

Table 3-6. Pyruvate Kinase Isozymes M1/M2 Student's *t*-test distribution values.

Pyruvate kinase isozymes M1/M2 is down-regulated because at 1 hour, 24 hours and 72 hours the average %volumes of 2 Gy, 4 Gy and 7 Gy samples were lower than the controls and LPS. In addition, the Student's *t*-test for 2 Gy, 4 Gy and 7 Gy at 1 hour and 24 hours showed significant protein changes. The largest decrease in average %volume for the IR samples is at 24 hours, below 0.4 while at 72 hours the 2 Gy, 4 Gy and 7 Gy samples start to increase in %volume due to the body recovering. The average %volume and the Student's *t*-test values of the LPS samples is not significantly different than the controls at all three time points.

The last protein that is a down-regulated biomarker is ATP synthase subunit beta, mitochondrial precursor according to the average %volume (Figure 3-11a to Figure 3-11c) and the Student's *t*-test values (Table 3-7).

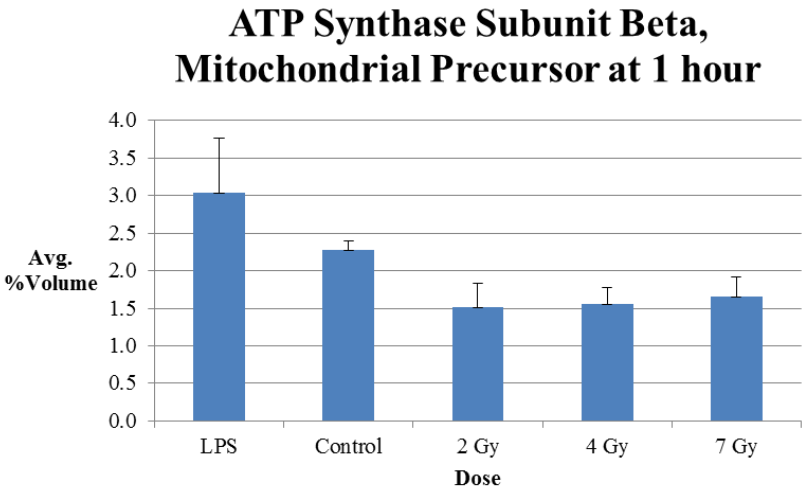


Figure 3-10a. The Average %Volume of ATP Synthase Subunit Beta, Mitochondrial Precursor as a function of radiation dose in comparison to control at 1 hour.

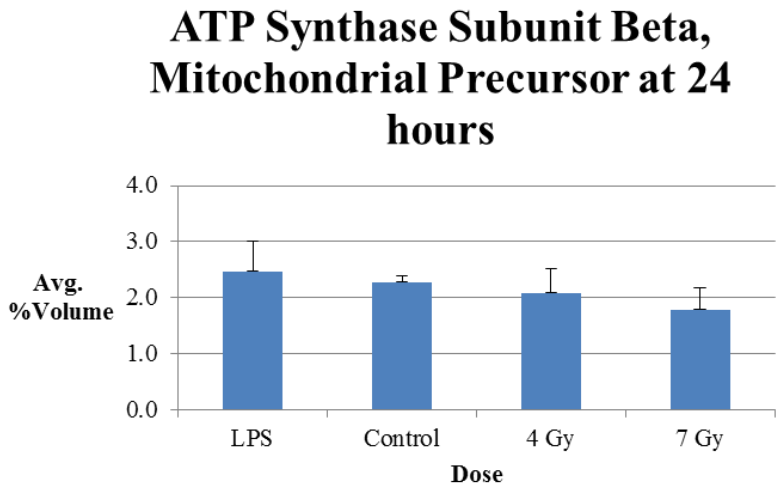


Figure 3-10b. The Average %Volume of ATP Synthase Subunit Beta, Mitochondrial Precursor as a function of radiation dose in comparison to control at 24 hours.

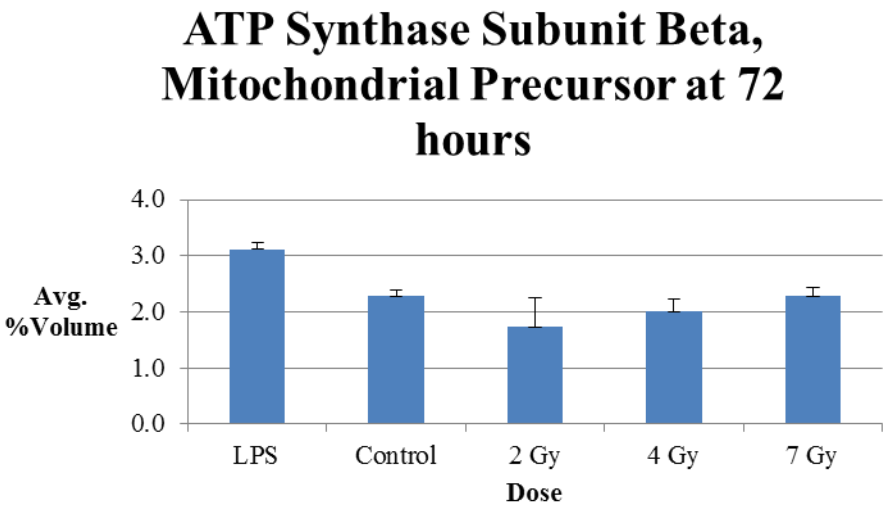


Figure 3-10c. The Average %Volume of ATP Synthase Subunit Beta, Mitochondrial Precursor as a function of radiation dose in comparison to control at 72 hours.

ATP synthase subunit beta, mitochondrial precursor [Mus musculus]			
Student's *t* -test values	1 hour	24 hours	72 hours
Control vs. LPS	1.78	0.64	8.79
Control vs. 2 Gy	3.91		1.73
Control vs. 4 Gy	4.99	0.73	1.88
Control vs. 7 Gy	3.72	2.01	0.09

Table 3-7. ATP Synthase Subunit Beta, Mitochondrial Precursor Student's *t*-test distribution values.

ATP synthase subunit beta, mitochondrial precursor is down-regulated because at 1 hour the average percent volumes of 2 Gy, 4 Gy and 7 Gy were significantly lower than the controls and LPS. Also, the Student's *t*-test values display a significant protein change at 1 hour. At 24 hours this protein appears to recover indicating that ATP synthase subunit beta, mitochondrial precursor is a potential biomarker for only 1 hour since at 24 hours and 72 hours the %volume of this protein is not different than the controls and there is no significant change from the Student's

t-test values. The average %volume for LPS is consistently higher than the controls for all three time points but it is only significantly higher at 72 hours.

CHAPTER 4

DISCUSSION

4.1 Using 2D Gel Electrophoresis, MALDI TOF-TOF and Quantification to Test Hypothesis

The hypothesis of this project was that when a mouse is exposed to whole body ionizing radiation it causes an increase or decrease in specific protein concentrations in the tongue of the mouse. The tongue was chosen because it is one of the radiosensitive organs in the body [41]. This was tested by exposing mice to TBI at doses of 2 Gy, 4 Gy and 7 Gy and sacrificing them at times of 1 hour, 24 hours and 72 hours. Mice injected with LPS were also sacrificed at 1 hour, 24 hours and 72 hours. The purpose of using LPS was to cause an inflammatory response in mice to see if that would affect the concentration of the same proteins in the tongue. A series of control experiments with BCA demonstrated that the experimental procedures could identify changes in protein concentration and correctly identify a protein.

From the results, five proteins were identified as biomarkers where one was up-regulated while the other 4 were down-regulated. The comparison of the %volumes of these proteins to the controls indicate that the hypothesis was correct in that whole body radiation does cause significant changes in protein concentrations in the tongue. The results also show that LPS, which was given to induce an inflammatory response, resulted in up-regulation or no significant changes in all of the protein biomarkers identified in this work when compared to the controls [18].

4.2 The Protein Spike Bovine Carbonic Anhydrase Structure and Function

BCA was chosen for the controlled spiked experiment because it was closest to malate dehydrogenase in isoelectric point and molecular weight. Malate dehydrogenase was originally

thought of as a protein biomarker because it was consistently identified in the radiated tongue samples. Also, the function of malate dehydrogenase is to convert malate to oxaloacetate by reversibly catalyzing the reaction and reducing NAD^+ to NADH. This reaction is in the tricarboxylic acid (TCA) cycle which produces pyruvate as an end product to be transferred to the mitochondria to make ATP [42]. It was believed that malate dehydrogenase would be either an up-regulated or down-regulated protein biomarker because it was a protein located in the mitochondria. Typically, reactive oxygen species (ROS) are made in the mitochondria which can disrupt the enzymes involved [43].

However, when analyzing the results on ImageMaster the average percent volume vs. dose did not display an up-regulated or down-regulated protein of IR exposure (was not statistically significant from the controls). Despite that, BCA was still used for the control experiments. BCA (Figure 4-1) is an enzyme that catalyzes the conversion of water and carbon dioxide to protons and bicarbonate which keeps the acid-base ratio stabilized in blood and tissues. BCA in general helps to remove carbon dioxide out of tissues. BCA is primarily found in skeletal muscle and the kidneys [44].

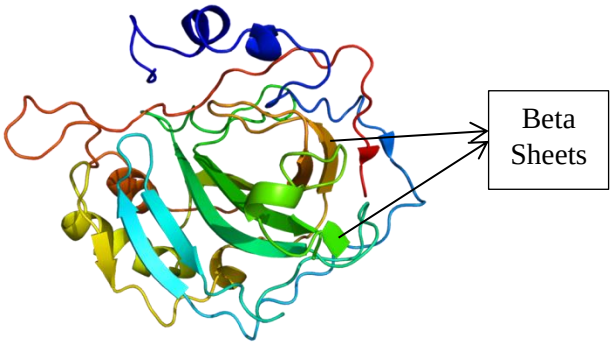


Figure 4-1. Protein Structure of BCA. Figure adapted from reference [45].

The BCA structure is composed of beta proteins, seen as sheets. BCA also has ligands, co-factors, ions and modified amino acids. At the center of the structure, there are hydrophobic properties that bind covalently with two ligands, zinc and mercury (II) ion. The amino acid that primarily binds to zinc is histidine and the amino acid that binds to mercury is cysteine [45].

In the BCA spiked experiments the results show decreasing average %volume with decreasing BCA spike (Table 4-1).

BCA Experiment 1	Average %Volume	Standard Deviation		Student's *t* -test values	
290 µg (n=2)	0.145	0.001		Control vs. 290 µg BCA	78.17
145 µg (n=2)	0.090	0.008		Control vs. 145 µg BCA	24.07
72.5 µg (n=3)	0.054	0.002		Control vs. 72.5 µg BCA	42.78
Control (n=3)	0.038	0.009			
BCA Experiment 2	Average %Volume	Standard Deviation		Student's *t* -test values	
290 µg (n=2)	0.196	0.003		Control vs. 290 µg BCA	13.41
145 µg (n=2)	0.150	0.006		Control vs. 145 µg BCA	6.46
72.5 µg (n=3)	0.108	0.038		Control vs. 72.5 µg BCA	0.37
Control (n=3)	0.102	0.016			

Table 4-1. BCA average percent volumes, standard deviation and Student's *t*-test values for experiments 1 and 2.

Also, the standard deviation for the BCA replicates was small, less than 0.017 except for the 72.5 µg BCA from the second experiment because there was an outlier from 72.5 µg BCA (C) which raised the percent relative standard deviation for this group to 0.038. The Student's *t*-test for each BCA amount was above the threshold which is 2.571 except for 72.5 µg BCA from experiment 2. This is due to the large amount of artifacts seen in the gel image. The majority of those artifacts were removed before running the comparison but it is likely that some were missed and

included in the %volume result. However, other than this one spike, the BCA control experiments were successful.

4.3 Protein Biomarkers Function and Structure Describing Up or Down-Regulation

As noted in the results section, aconitate hydratase, mitochondrial precursor was a protein biomarker identified as having different concentrations between the irradiated and control samples. The molecular weight is 86,151 Da (molecular weights from all of the biomarkers were found by the Mascot search engine). It is an enzyme that catalyzes citrate to isocitrate by cis-aconitate in the TCA cycle. The function of the TCA cycle is to make ATP [46].

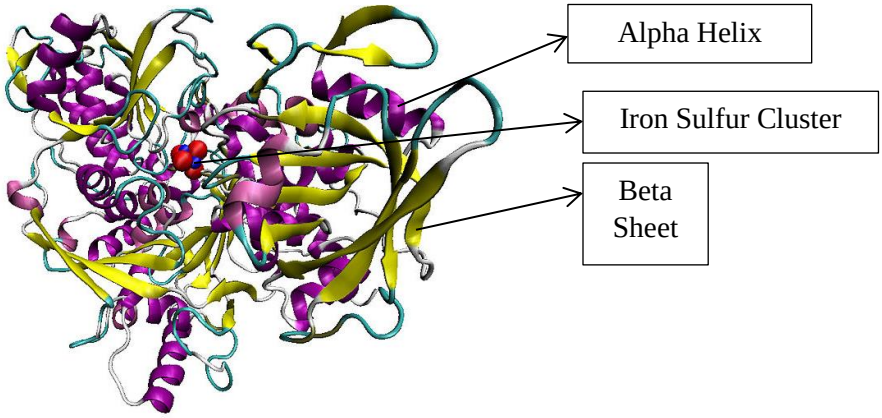


Figure 4-2. Protein Structure of aconitate hydratase, mitochondrial precursor. Figure adapted from reference [47].

The protein is composed of alpha helices and beta sheets (Figure 4-2). The molecules in the center are an iron sulfur cluster. When aconitate hydratase is activated, the cluster is in the $[Fe_4S_4]^{2+}$ form which is converted to $[Fe_3S_4]^{+}$ when inactivated. Three cysteine (Cys) residues assist in the formation of the iron sulfur cluster center [47]. When observing the average percent volume vs. dose, dependent of time, aconitate hydratase displayed a decrease in percent volume in comparison to the controls. This would be classified as a down-regulated protein. The down-

regulation would make sense because the iron-sulfur cluster is very sensitive to superoxide molecules which are produced in the interaction of ionizing radiation with water in cells. Once the superoxide is made, it could penetrate the iron-cluster center of aconitate hydratase, disrupting the structure due to its sensitivity. This disruption causes a decrease in activity and thus concentration.

The next protein identified and analyzed is alpha-actinin-2. It has a molecular weight of 104,339 Da and is an actin-binding protein with many roles, depending on the cell type. For instance, in non-muscle cells, alpha-actinin-2 assists in binding actin to the membrane at the cytoskeletal isoform cite. However, for muscle cells from skeletal, cardiac, and smooth muscle tissue, alpha-actinin-2 assists in pulling the actin filaments in the Z-disc that help with contractions for muscle movement [48].

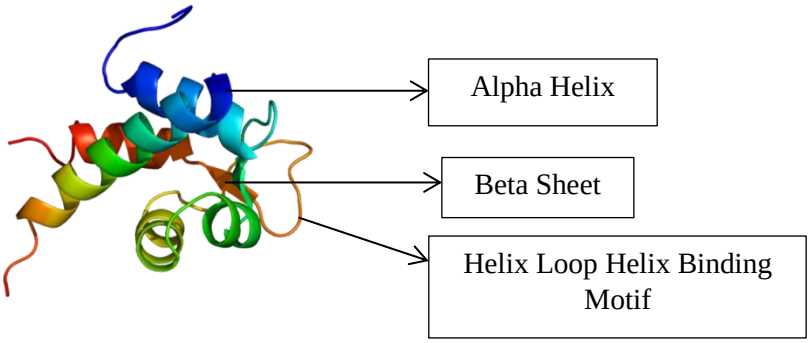


Figure 4-3. Protein Structure of alpha-actinin-2. Figure adapted from reference [49].

Alpha-actinin-2 (Figure 4-3) is composed of four alpha helices and a short beta sheet with a helix loop helix binding motif. This protein is a common EF-hand domain which can hold up to two calcium ions. The alpha-actinin-2 is only dependent on calcium for non-muscle cells. For muscle cells, alpha-actinin-2 is dependent on the interaction of binding to titin which is a bulky protein,

at 3,000 Da for muscle movement. Titin interacts with alpha-actinin-2 to help with connecting the Z-line to the M-line for muscle contraction.

This is done when titin forms a helical structure over 18 residues (Lys 655–Arg 673) which binds between the helix pairs of alpha-actinin-2 inside the hydrophobic pocket. Specifically, the hydrophobic side chains of titin which are amino acids alanine and valine side chains connect with the hydrophobic center alpha-actinin-2. The important interaction is the four valine residues of titin. Additional interactions can happen between a set of hydrophilic residues from positively charged groups of lysine 656 and arginine 671 from titin which are attracted to the negatively charged side chains of glutamine 853 and aspartate 857 from alpha-actinin-2 and can make salt bridges between them [49].

From the observed average percent volume vs. dose, alpha-actinin-2 is a down-regulated protein. This would make sense because it is located in muscle tissue of the tongue. Also, when tissue is irradiated, the ablated area causes a decrease in α-actinin and the density of actin filaments in the entire cell cortex which induces an acceleration of cytokinesis [50].

The third protein discussed is troponin I, skeletal, fast 2 (Figure 4-4). The skeletal fast protein is a subunit of troponin I which is a branch of the troponin complex [51]. The function of troponin one is to bind to actin to keep the actin-tropomyosin complex intact so myosin does not bind to actin when the muscles are relaxed. Troponin I does not bind to actin when the muscles are contracting. This mechanism happens when calcium binds to troponin C (also part of the troponin complex) which changes its structure. This alteration releases troponin C from troponin I. Then troponin I unbinds to actin which allows myosin to bind to actin during contractions [52].

The isoform of troponin I is in the fast-twitch skeletal muscle. The molecular weight is 20,954 Da.

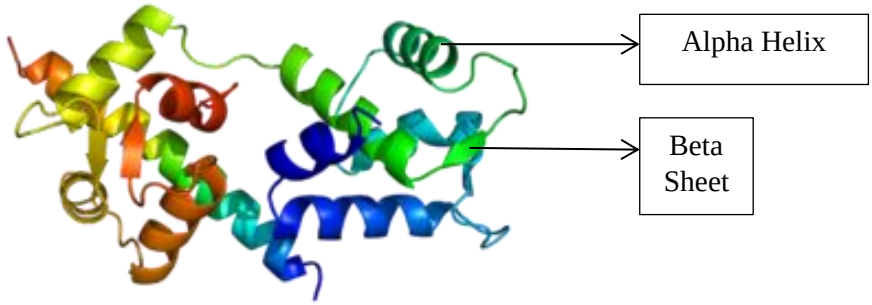


Figure 4-4. Protein Structure of troponin I, skeletal, fast 2. Figure adapted from reference [53].

Troponin I, skeletal fast 2 is composed primarily of alpha helices and some beta sheets. The measured average percent volume vs. dose, dependent on time, shows that troponin I, skeletal fast 2 is an up-regulated protein. Troponin I, skeletal fast 2 is the only protein biomarker found to be up-regulated in this project. The reason for the up-regulation is that radiation exposure can cause severe inflammation, especially in radiosensitive organs such as the tongue. Whenever there is inflammation, there is an increase in troponin I, skeletal fast 2, which combats muscle cell death.

The fourth protein identified is pyruvate kinase isozymes M1/M2, also called PKM2 or pyruvate kinase muscle isozyme (PKM). This protein's molecular weight is 58,378 Da. The primary function of PKM is as an isoenzyme to convert phosphoenolpyruvate to pyruvate in glycolysis. The importance of pyruvate is it goes through the TCA cycle and produces ATP [54]. PKM1 and PKM2 are both isoenzymes, but they are spliced differently in the M-gene [55]. Another difference is that PKM1 and PKM2 are expressed in different organs. PKM1 is expressed in organs that need an abundance of energy such as muscles and brain [56]. Whereas PKM2 is

expressed in multiple tissues including lung, fat, retina, pancreas and in any cells that have a high rate of nucleic acid synthesis [57].

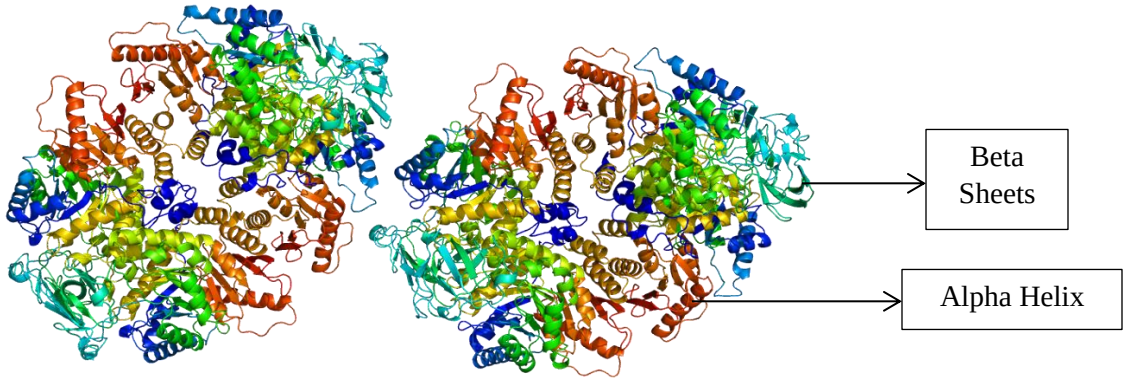


Figure 4-5. Protein Structure of pyruvate kinase isozymes M1/M2. Figure adapted from reference [58].

Pyruvate kinase isozymes M1/M2 structure is composed of alpha helices and beta sheets (Figure 4-5). It also has binding sites where it binds to potassium and magnesium in order to convert phosphoenolpyruvate to pyruvate [58]. This isozyme was determined to be a down-regulated protein biomarker from the results of the average percent volume vs. dose, dependent on time. This is the only protein biomarker identified in this work where the results conflict with the literature. Normally, when cells are damaged from ionizing radiation, there is an increase in ATP production to repair cells and prevent apoptosis thus this protein should be up-regulated instead of down-regulated. The best explanation for it to be down-regulated is reactive oxygen species caused by IR damage inhibits pyruvate kinase M2 by oxidation of cysteine 358 [59].

The last protein identified as a biomarker is ATP synthase subunit beta, mitochondrial precursor. The protein's molecular weight is 56,265 Da. The function of this protein is to accelerate ATP

synthesis which is done during oxidative phosphorylation by using an electrochemical gradient of protons from the inner membrane to generate ATP [60].

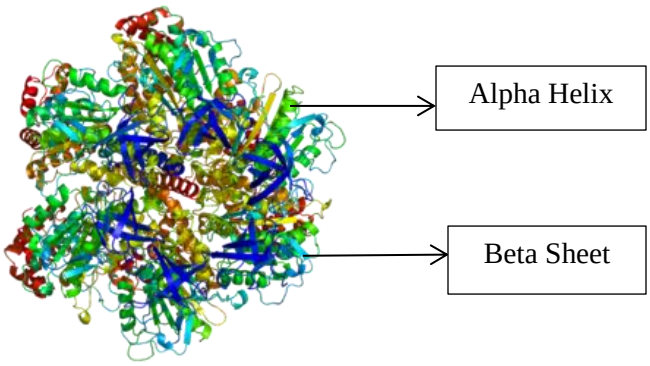


Figure 4-6. ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide. Figure adapted from reference [61].

This structure has five subunits which are alpha, beta, gamma, delta and epsilon (Figure 4-6). The one discussed has the beta subunit (with beta sheets displayed) which is part of the core that catalyzes ATP synthesis [60]. ATP synthase subunit beta, mitochondrial precursor is down-regulated from the average percent volume vs. dose, dependent on time. This makes sense because ionizing radiation produces ROS from the mitochondria. Those free radicals disrupt the mitochondria leading to apoptosis and mutations.

4.4 Protein Biomarkers and How Their Structure and Functions are affected by IR

The proteins identified were either involved in muscle movement, glycolysis or assisting in producing ATP in the mitochondria. The proteins found in muscle tissue would be affected by ionizing radiation. The protein involved in glycolysis would be down-regulated due to ROS free radicals inhibiting it. Proteins found in the mitochondria, were down-regulated because of ROS free radicals that are produced in the mitochondria attacking it, and thus disrupting its structure.

CHAPTER 5

FUTURE DIRECTIONS

5.1 Protein Biomarkers of Ionizing Radiation Exposure

Though five proteins were identified by running 2D gel electrophoresis and MALDI TOF-TOF, this project needs to be repeated. In part because in this project the results from mice radiated at 2 Gy and sacrificed at 24 hours are not present due to a miscalculation in how much protein was needed to be loaded for gel separation. There would need to be a new triplicate of mice exposed to TBI of 2 Gy, 4 Gy and 7 Gy and sacrificed at 1 hour, 24 hours and 72 hours. In addition, a new triplicate of controls and LPS injected mice sacrificed at 1 hour, 24 hours and 72 hours. Then their tongues harvested where they go through sample processing, isoelectric focusing, 2D gel electrophoresis, quantification and MALDI TOF-TOF. However, this time these proteins can be excised from every gel to make the results more consistent. In this project the spots were not excised consistently because at first they were not quantified correctly.

In ImageMaster the spots were incorrectly matched and had to be redone to get the right values for percent volume and the Student's *t*-test. Another change is that all of the mice would be weighed before and after they were radiated. The reason is to see if the mice have a physical change after radiation exposure and not just a chemical one. In addition, all of the mice tongues will be weighed before they went through sample processing. The tongues in this project were not weighed prior to them going through sample processing, isoelectric focusing, 2D gel electrophoresis, quantification, trypsin digestion and MALDI TOF-TOF. The assumption was that in the sample processing step calculating how much each protein needed to be diluted to be

loaded for separation normalized the concentration. But to check that there was an equal amount of tissue sample the mice tongues need to be weighed.

Besides the experiment having to be repeated, to really know if LPS affects these biomarkers mice need to be injected with LPS and radiated at 2 Gy, 4 Gy and 7 Gy where they would be sacrificed at 1 hour, 24 hours and 72 hours. Then their tongues harvested and weighed where they would go through the same experimental methods as this project. The mice from the repeated experiment and the LPS injected TBI mice would go through quantification to determine if these proteins are still up-regulated or down-regulated by average percent volume vs. dose by time of sacrifice. Finally, those protein spots would be excised from all of the gels and go through trypsin digestion and MALDI TOF-TOF to check their identifications. This would show more consistent results for these protein biomarkers of IR.

5.2 Standardization

Another task that would have to be done is to produce another standard response curve. Although the BCA spike experiments were successful in demonstrating a proportional response of average percent volume to BCA spike, a more effective standard curve could be produced by using a protein not found in mice. The issue with using BCA as a standard is that it was a substitute for malate dehydrogenase which was incorrectly identified as a protein biomarker. If a spike is used that is not in mice then when quantifying it by ImageMaster the average percent volume of a control would only be background which would make the calculations easier since that value would be next to zero. The controlled experiment for the new spikes should be repeated for consistency.

5.3 Alternative Methods to Identify Protein Biomarkers and Their Statistical Significance

An alternative method to identify protein biomarkers of IR exposure is to take freeze dried tongue samples and homogenize them in methanol with water. Then to induce precipitation, ACN is added where the samples are chilled in ice and centrifuged. To get the samples in a pellet, the samples will be dried under vacuum (lyophilized). Then the pellet should be resuspended in a radioimmunoprecipitation assay buffer (RIPA) chilled on ice and centrifuged for UPLC-QTOF-MS for protein analysis and identification [16]. This method could be used because in the literature UPLC was known to generate fast results and reproduce them with little sample consumed [62].

In this project the Student's *t*-test was used to determine protein differences between IR samples and controls to determine biomarkers of IR exposure. However, numerous references show that the significance of a protein biomarker increases when a combination of those biomarkers is tested. To test the five identified biomarkers, and future biomarkers, the Wilks' lambda (F test) will be performed for different combinations. The Wilks' lambda will test the mean of sample groups such as controls, LPS, 2 Gy, 4 Gy, and 7 Gy in addition to LPS injected IR mice, to see if there are differences by subjecting them to a combination of variables [63]. The advantage of using the F test is it tests the samples collectively to see if there is any significance. If there is any significance then the F test analyzes which combination of samples, in comparison to the controls, has the highest discrimination power and thus most significant [12].

5.4 Developing a Lateral Flow Device

Once the protein biomarkers of IR exposure project have been completed by repeating experiments and running new ones with LPS injected TBI mice. If the same protein biomarkers

have been identified and through quantification they show to be up-regulated or down-regulated then a lateral flow device can be made. This would happen by purchasing an antibody of the protein biomarker and impregnating it to a color test strip. This would then be tested on mice that will be radiated with 2 Gy, 4 Gy and 7 Gy. Also, control mice, LPS injected mice and LPS injected mice that received TBI of 2 Gy, 4 Gy and 7 Gy.

The lateral flow device would be used on the mouse's tongue at 1 hour, 24 hours and 72 hours to see if the test strip worked. If it produces a color that indicates the mouse has been exposed to 2 Gy or higher and that specific biomarker had a change in protein regulation/concentration. If there is no change in color then that mouse was exposed to less than 2 Gy. If the test strip works then the next step would be to test people who received radiation for head and neck injuries and people who were not radiated as a control.

The lateral flow device would rub the person's tongue to see if the test strip showed a color response. Again, if the lateral flow device changes color it indicates that a person has been exposed 2 Gy or higher and if the test strip does not change color then that person has been exposed to less than 2 Gy. If the lateral flow device experiment works on people then it can be used to triage victims of a radiological dispersal device or nuclear detonation.

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