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John C. Stagg
Georgia Southern University

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Assessment of Oxidative Stress in the Livers of Rats Exposed to 2-Aminoanthracene for Twelve Weeks

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

April 2019

By

John C Stagg

Under the mentorship of Dr. Eric W Gato

Abstract

Diabetes is a chronic illness that affects many people, and it's thought that environmental exposure to certain compounds may increase its proliferation. In this study, we examine the dietary effects of 2-aminoanthracene (2AA), a polycyclic aromatic hydrocarbon (PAH), in rats, specifically as it relates to oxidative stress. Oxidative stress refers to the imbalance of reactive oxygen species (ROS) and antioxidants in living systems; it has been shown to contribute to type-1 diabetes. To accomplish the study objectives rats were divided into three dietary treatment groups, control (0 mg/kg-2AA), low dose (50 mg/kg-2AA) and high dose (100 mg/kg-2AA). Rats were fed 2AA for three months, and then euthanized using carbon dioxide. Various tissues including the liver and were flash-frozen in liquid nitrogen. To determine 2AA effects in the liver, a portion of the liver was homogenized in phosphate buffered saline solution. The supernatant was analyzed for serum albumin, aspartate aminotransferase (AST), IgA protein, and antioxidants. Furthermore, genes such as Pdx1, Sod1, Gpx1, Prdx6, Ncf2, Duox1 and Ptgs2 typically used to measure cellular oxidative stress were quantified. The expressions of these genes in the liver show the upregulation of Sod1 and Prdx6, and downregulation of Ptgs2 in treated groups. AST activity was marginally increased in treated groups while the albumin concentration was elevated in the 50 mg/kg group and reduced in the 100 mg/kg-2AA animals. IgA levels were slightly elevated while antioxidant levels were reduced in treated groups. Taken together, these results show that ingestion of 2AA may produce hepatic oxidative stress.

Acknowledgements

I would like to thank Raven Ulieme, Bethany Bogan, and Carter Mixson; undergraduate students who assisted me with experimentation in this study. I am grateful to Craig Banks, the director of the Georgia Southern University Animal Care Facilities, and the staff and volunteers of this facility who housed and cared for the animals during the animal study portion of this project. I am especially indebted to Dr. Eric Gato who mentored and guided me through this project, and for allowing me to use his lab as a hub for my work. This project was partially supported using the Faculty Seed Grant from Georgia Southern University. This project was also funded through support from the Georgia Southern University Honor's College. Without these people and organizations this project would not have been possible.

Background and Objective

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds produced during the incomplete combustion of hydrocarbons. They can also be formed in high pressure reactions involving hydrocarbons. People are exposed to PAHs everyday through many sources such as natural sources (forest fires, volcanos, etc.), cigarette smoke, vehicle exhaust [Cherg, Wetering, Talsma, Crommelin, & Hennink 1996; Lewitas 1997], and diet. In nonsmokers up to 70% of PAH exposure occurring outside of the workplace is associated with the diet [Skupinska, Misiewicz, & Kasprzycka-Guttman 2004]. This project will focus on diet as the point of exposure.

PAHs are known to be carcinogenic. One especially toxic class of PAHs is anthracenes which are PAHs with the chemical formula $C_{14}H_{10}$. Anthracenes are classified as group of top concern by the United States Agency for Toxic Substances and Disease Registry (ASTDR) due to their toxicity to humans which is by comparison greater than that of other PAHs. Research has shown 2-aminoanthracene (2AA) to affect genes involved with carbohydrate and lipid metabolism, as well as inflammation and immune responses [Wilson, Buckeridge, Yau, Howerth, & Gato 2017]. These effects have led to symptoms of diabetes in rats.

Just like other PAHs 2AA is mildly carcinogenic. According to previous research exposure to mild carcinogens can cause oxidative stress [Takehashi, Wei, Fukushima, & Wanibuchi 2013], and oxidative stress has been shown to be a factor that may contribute to type-1 diabetes [Mays, Hunter, Wilson, & Gato 2017]. Oxidative stress is a phenomenon that occurs when the balance of reactive oxygen species (ROS) to antioxidants in the body is disturbed. Reactive oxygen and nitrogen species are constantly

being produced through processes such as cellular respiration. These reactive species are harmful to the body. They can cause inflammation and damage to the cells. Over time this can lead to cancerous growth in the cells. Enzymatic antioxidants are needed to break down the reactive species.

In this project we examine the livers of rats that have been exposed to a diet containing 2AA. We hypothesized that rats exposed to 2AA for 12 weeks will demonstrate oxidative stress in their liver cells. This study's aim was to provide us with information about the associated risk of type-1 diabetes from exposure to 2AA. In addition to the damage and inflammation we expected to observe from oxidative stress we also expected to see inhibition of carbohydrate and lipid metabolism [Wilson et al. 2017]. This information is useful because it provides information about the risk humans assume from consuming 2AA which can be present in food products. The effects of 2AA on the liver was measured through analysis of serum isolated from liver samples for albumin levels, aspartate aminotransferase (AST) activity, IgA protein levels and antioxidant levels. Furthermore, analysis of specific genes related to oxidative stress was also carried out. The genes that were quantified in the study included Pdx1, Sod1, Gpx1, Prdx6, Ncf2, Duox1 and Ptgs2.

Materials and Methods

Animal Study

The rats used in this project were Male Sprague Dawley obtained from Taconic Bioscience Inc. (Hudson, NY). 2AA was purchased from Sigma Aldrich (St. Louis, MO). The diet used for this project was The Global Rodent Diet 2020. This formula was

obtained from Harlan Laboratories Inc. (Madison, WI). 2AA was sent to Harlan Laboratories and incorporated into some of the diet (all portions besides control).

During the feeding portion of the experiment the rats were housed at the Georgia Southern University Animal Facility. The rats were fed three different dose regimens 0 mg/kg (control), 50 mg/kg (low dose-LD), and 100 mg/kg (high dose-HD). The rats were fed the diet for 12 weeks and were then sacrificed using carbon dioxide. The kidneys and livers of the animals were harvested and measured, and a blood sample was collected. Discomfort of the Sprague Dawley rats was minimized through treatment methods outlined in the Institute for Laboratory Research (ILAR) Guide for Care and Use of Laboratory Animals. All measures and protocols were approved by Institutional Animal Care and Use Committee (IACUC) prior to beginning the experiment.

Homogenization of Hepatic Tissues

In order to evaluate 2AA effects directly on the liver, 20-30 mg of each hepatic tissue from each sample was homogenized in 1X PBS (Fisher Scientific, Hampton NH, Cat#: BP399). Samples were then centrifuged at 4°C for 15 minutes. The supernatant was aliquoted and saved in -80°C freezer until analysis.

Quantitative Determination of Serum Albumin

The concentration of serum albumin in the homogenized liver samples was determined using the Albumin Reagent Set (Pointe Scientific Inc., Canton, MI, Cat#: A7502-1L). In this experimentation bromocresol green dye was bound to albumin, and the absorbance was measured at 630 nm using a UV-Vis spectrometer in accordance with

the manufactures guidelines. A standard curve was created using Bovine serum albumin (BSA) to quantify the data collected.

Quantitative Determination of Aspartate Aminotransferase (AST)

Aspartate aminotransferase (AST) activity was measured using the AST (SGOT) Liquid Reagent Set (Pointe Scientific Inc., Canton, MI, Cat#: A7561-450). This assay functions by the principle that AST catalyzes and transfers the amino group from L-aspartate to α -Ketoglutarate to yield oxalacetate and L-glutamate. In a subsequent reaction the oxalacetate as NADH is oxidized to form NAD^+ . As this reaction progresses the rate of decrease of absorbance measured at 340 nm is directly proportional to the AST activity. To measure the absorbance accurately lactate dehydrogenase (LDH) must be added to prevent endogenous pyruvate, which is typically found in serum, from interfering with the absorbance reading. The experiment was carried out as specified in the manufacturers guidelines. The following equation was provided by the manufacturer to calculate AST activity from change in absorbance per minute:

$$\text{AST (IU/L)} = \Delta\text{Abs./min} \times 1768$$

IgA Quantitative Analysis via ELISA

Using the Mouse IgA Ready-Set-Go! ® (Affymetrix eBioscience, Santa Clara, CA, Cat#: 88-50450) assay kit IgA protein levels in serum were quantified for each of the treatment groups using enzyme-linked immunosorbent assay (ELISA). The reaction was set up in a 96 well microplate, and the absorbance of the plate was measured at 450 nm using a microplate reader. The experiment was carried out according to the manufactures guidelines. A standard curve was created from mouse IgA provided with the kit. This

standard curve was then used to calculate the IgA concentrations for samples from our study.

Total RNA Isolation

Total RNA was isolated from liver samples using the RNeasy Mini by QIAGEN Total RNA Isolation Kit (QIAGEN N.V., Hilden, Germany, Cat#: 74104). The experiment was carried out using the guidelines stated in the protocol. Quality of the RNA was determined using gel electrophoresis. RNA Sample Loading Buffer (Sigma Aldrich, Saint Louis, MO, Cat#: R4268) was used for this process. The loading buffer was mixed at a ratio of 1 volume of sample to 2-5 volumes of RNA Sample Loading Buffer before being run in a gel. Excess isolated RNA was stored in a -80°C freezer for further analysis.

Analysis of Selected Genes

Gene Expression was carried out using quantitative real time polymerase chain reaction (qRT-PCR) for the oxidative stress specific genes Pdx1, Sod1, Gpx1, Prdx6, Ncf2, Duox1 and Ptgs2. FASTA mRNA sequences of the genes were generated from the National Center for the Biotechnology Information (NCBI). Using PrimerQuest by Integrated DNA Technologies (IDT) (Coralville, IA) forward and reverse primers for each of the mRNA sequences was obtained. The primers used are listed in Table 1. cDNA for use in RT-qPCR was synthesized using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, Cat#: 1708840). cDNA was diluted with 10 mM Tris-HCl buffer (pH 8), 01 mM EDTA prior to use in the qPCR system in accordance with the manufactures guidelines.

	Acession #	Forward	Reverse
PDX-1	U35632	CTGCCTTTCCCATGGATGAA	CTTGATGTGTCTCTCGGTCAAG
SOD1	KR710602	GCAGGTCCTCACTTAATCCTC	CTTCCAGCGTTTCCTGTCTT
GPX1	KR709800	CATCAGGAGAACGCCAAGAA	TGTCAGGCTCGATGTCAATG
PRDX6	KY383188	CCTCTGAAGGCTCCTGTTC	AGTAAGGCGCGGAATTT
PKC	KF705541	GTGCGGTCCTTGTCTTACTT	CCCATCCTCCTTGTCCATAATC
NCF2	KR709852	CTCCGGCTGGAACAACTAA	CTGGTTGGGTAGCCTCATAAC
DUOX1	GBYX01074484	TCATTGCCACGCTTTCT	CCTTCGATAAGTAGCCAGTTCC
PTGS2	KR709390	GCGGGAACACAACAGAGTAT	GGACAGCCCTCACGTTATT

Table 1. Forward and reverse nucleotide sequences used as primers for each gene.

Quantification of Total Antioxidant Capacity

Quantification of total antioxidants present in serum was determined using the Cayman Chemical Antioxidant Assay Kit (Ann Arbor, MI, Item#: 709001). This assay relies on antioxidants in the serum samples to inhibit the oxidation of 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS[®]) to ABTS^{®*+} by metmyoglobin. The suppression of ABTS^{®*+} production can be measured by the by reading the absorbance at 405 or 750 nm. In this experiment absorbance was measured at 405 nm. The absorbance at either 405 or 750 nm is inversely related to the proposed concentration of antioxidants in the sample. A standard curve was generated using Trolox, a water-soluble tocopherol analogue. The concentration of antioxidants is quantified as millimolar (mM) Trolox equivalents. The reaction setup was prepared in a 96 well microplate, and the absorbance of standards and samples was measure by a microplate reader. The reaction was set up in accordance with manufacturers guidelines.

Results

Quantitative Determination of Serum Albumin

A standard curve of absorbance vs. concentration of bovine serum albumin (BSA) was generated. This standard curve showed a strong linear relationship demonstrated by an r-squared value of 0.9837. The linear equation generated from this linear relationship was found to be $y = 0.8414x - 0.0146$. This relationship is summarized in Figure 1. This generated equation was used to calculate concentrations of serum albumin in the rat samples.

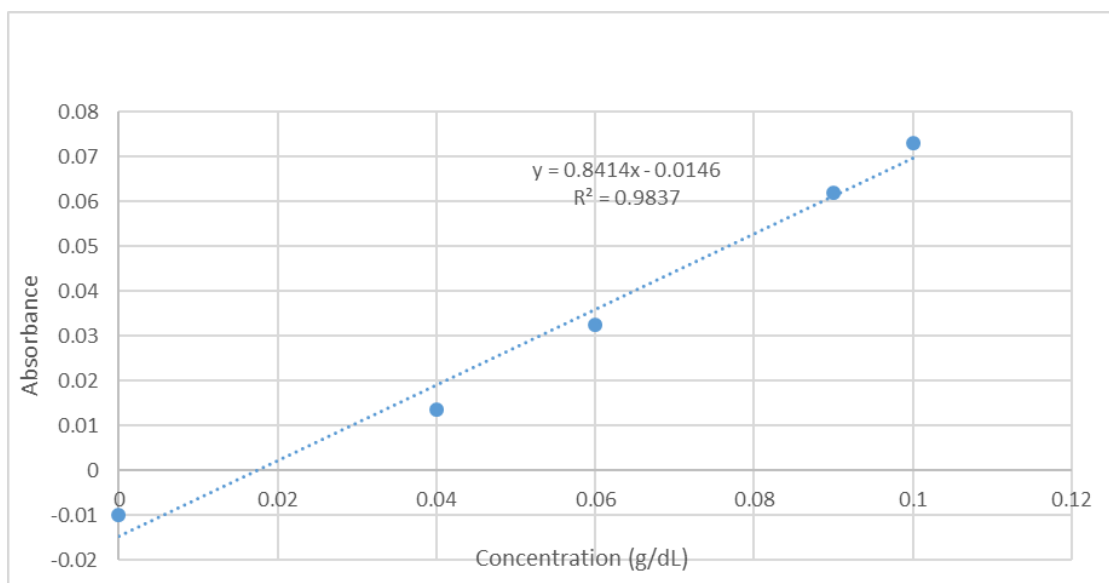


Figure 1. Standard curve of absorbance vs. concentration for bovine serum albumin (BSA).

Through the analysis of serum albumin levels in rats using the BSA standard curve it was found that animals in the low dose treatment group experienced a slight increase in serum albumin levels as compared to control animals; 0.9230 and 0.7933 g/dL respectively. Whereas animals in the high concentration group experienced a decrease in serum albumin levels. Serum albumin concentration in high dose animals was found to be

an average of 0.6037 g/dL. Each groups data is summarized in Figure 2. The standard error of each mean measurement is displayed in Figure 2.

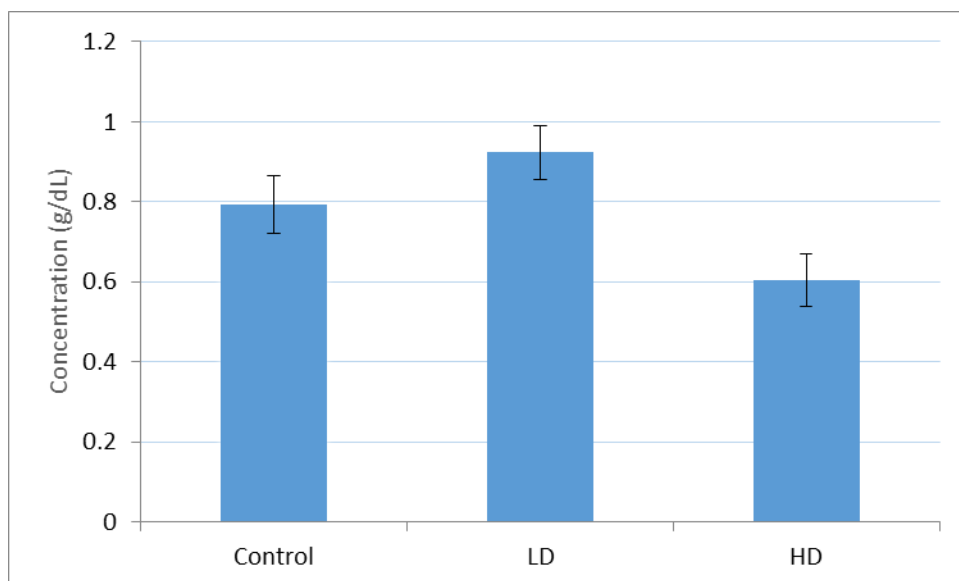


Figure 2. Mean albumin concentration from serum of rats in control (0 mg/kg-2AA), low dose (50 mg/kg-2AA), and high dose (100 mg/kg-2AA) treatment groups.

Quantitative Determination of Aspartate Aminotransferase (AST)

AST activity was measured and found to be slightly elevated in rats that had been fed 2AA. Looking at specific numeric values AST activity was measured to be an average 106.28 IU/L for the control group, 137.31 IU/L for the low dose group, and 130.54 IU/L for the high dose group. While AST activity was elevated in both treated groups it was slightly higher in the low dose group. These results are summarized in Figure 3. The standard error for this measurements is also visualized in Figure 3.

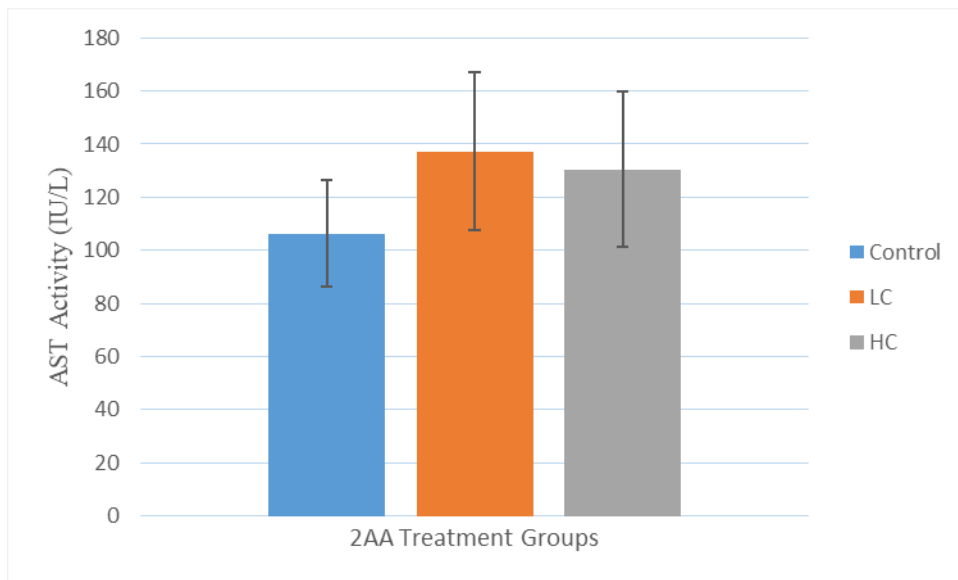


Figure 3. Mean AST activity levels from serum of rats in control (0 mg/kg-2AA), low dose (0 mg/kg-2AA), and high dose (100 mg/kg-2AA) treatment groups.

IgA Quantitative Analysis via ELISA

A standard curve of absorbance of standard mouse IgA at 450 nm vs. concentration of standard mouse IgA was created. This curve showed strong linearity with a generated linear equation of $y = 0.0044x + 0.0839$. This equation was used to calculate the concentration of IgA in our rat serum samples. The linear relation found from the standard mouse IgA is summarized in Figure 4.

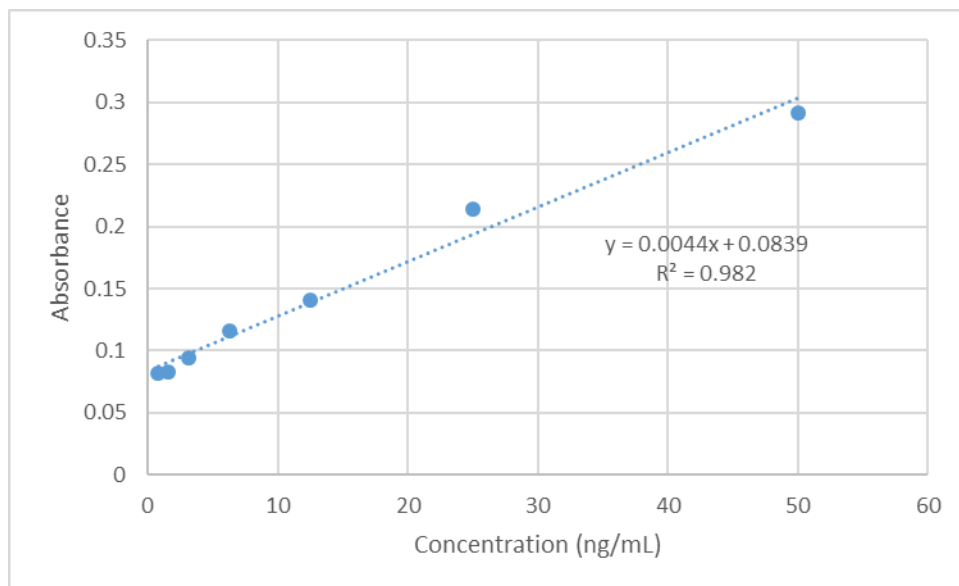


Figure 4. Standard curve of absorbance vs. concentration of mouse IgA.

Using the generated standard curve concentrations of IgA in the serum samples the concentrations of IgA were calculated in ng/mL for each treatment group. The concentration for that of the low dose group was statistically insignificant from the control while the concentration of the high dose treatment group showed slightly elevated levels, though this elevation is also relatively statistically insignificant. The average concentrations of the low dose and high dose groups were 136.22 and 148.55 ng/mL respectively. The average concentration of IgA in the control treatment group was 133.41 ng/mL. These findings are summarized in Figure 5.

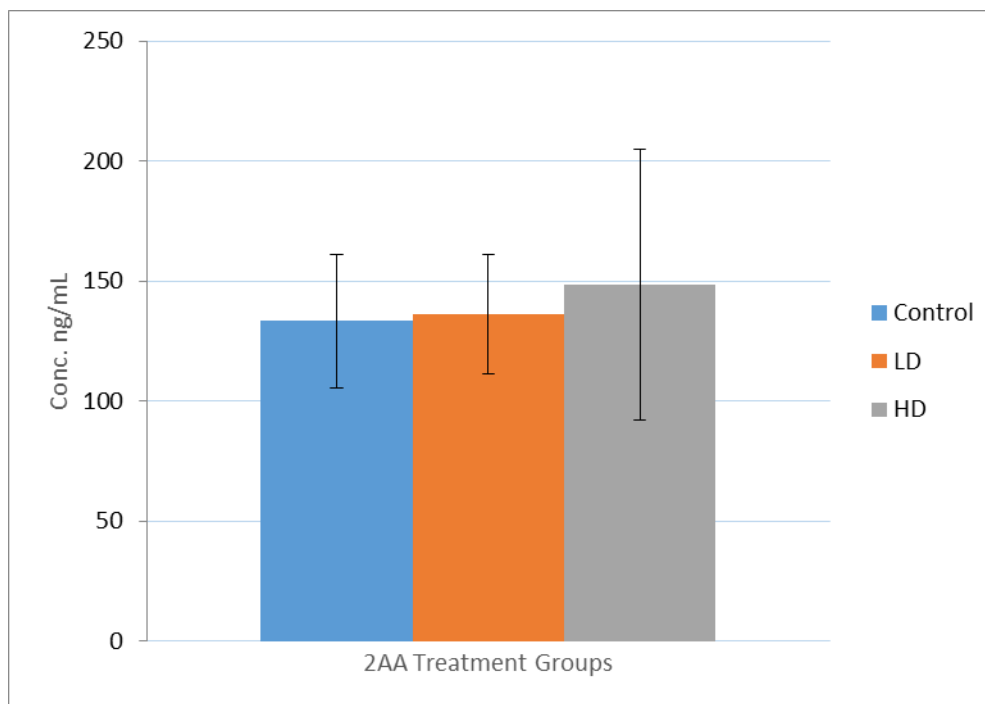


Figure 5. Mean IgA levels from livers of rats for control (0 mg/kg-2AA), low dose (50 mg/kg-2AA), and high dose 2AA (100 mg/kg-2AA) treatment groups.

Analysis of Selected Genes

At the conclusion of PCR analysis relative quantities of each gene were determined along with their standard deviation in the real-time PCR measurements. The expression of these genes showed upregulation of Sod1 and Prdx6, and downregulation of Ptgs2. The results of this experiment are represented graphically in Figure 6.

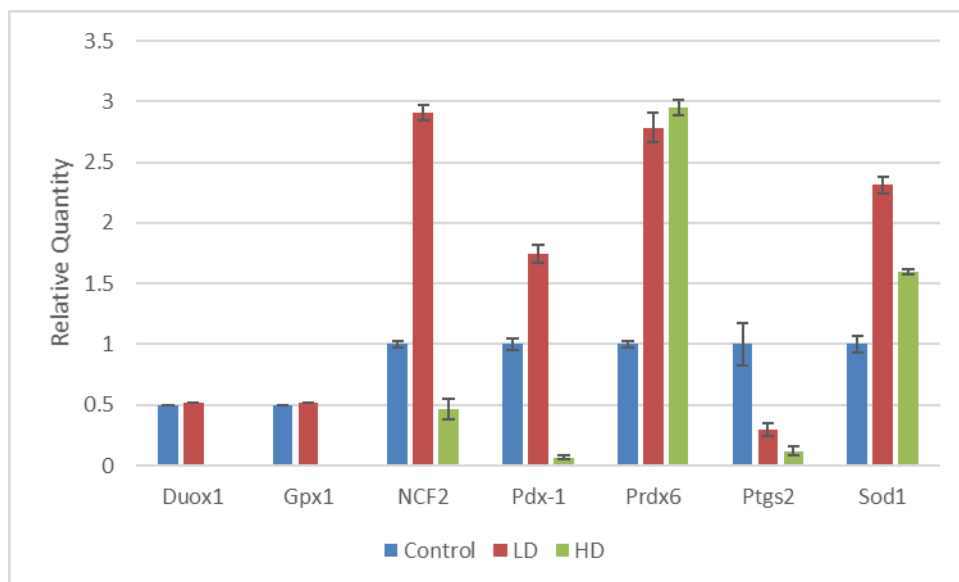


Figure 6. Relative gene expression for select genes (Duox1, Gpx1, Ncf2, Pdx-1, Prdx6, Ptgs2, and Sod1) in RNA extracted from the livers of rats. Rats were either in the control (0 mg/kg-2AA), low dose (50 mg/kg-2AA), or high dose (100 mg/kg-2AA) treatment groups.

Quantification of Total Antioxidant Capacity

A standard curve was generated using Trolox where an inverse relationship between absorbance and concentration was shown. The standard curve showed a strong linear relationship and generated a linear equation of $y = -1.5663x + 0.8309$. This equation was then used to quantify the concentration of antioxidant in the serum samples from each of the animal treatment groups. The linear relationship from the standard curve is visualized in Figure 7.

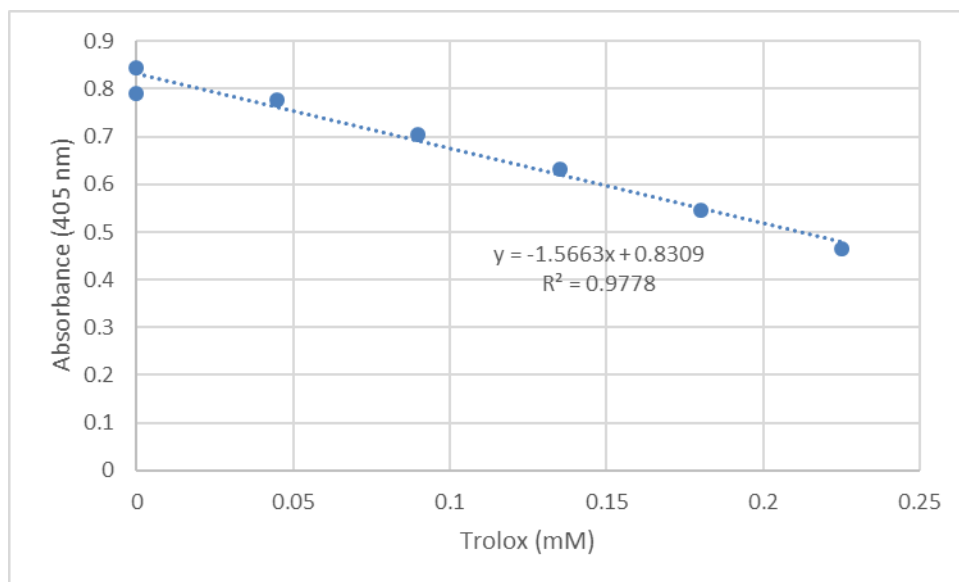


Figure 7. Standard curve generated from Trolox when absorbance was measured at 405 nm.

The antioxidant concentration as millimolar Trolox equivalents was determined and it was found that antioxidant concentration was decreased in treated animals. Low dose treated animals showed lower antioxidant concentrations than high dose treated animals, but both groups demonstrated a decrease in antioxidant concentration as compared to the control group. The average concentrations for each group were found to be 0.2268 mM equivalents for the control group, 0.1673 mM for the low dose group, and 0.1877 mM for the high dose group. This information is displayed on Figure 8 along with the standard error for each measured group.

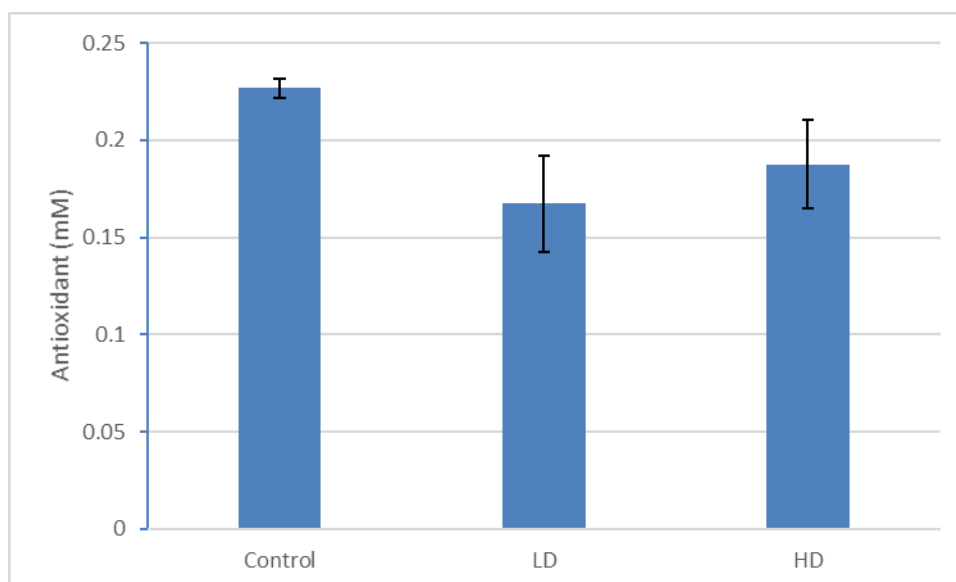


Figure 8. Inhibition of ABTS oxidation by antioxidants for control (0 mg/kg-2AA), low dose (50 mg/kg-2AA), or high dose (100 mg/kg-2AA) treatment groups.

Discussion

The primary objective of this study was to demonstrate oxidative stress in the livers of rats exposed to 2AA. Two very important markers when looking to identify oxidative stress are serum albumin levels and antioxidant capacity in body fluids [Marrocco, Altieri, & Peluso 2017]. Albumin is a protein that has shown antioxidant properties. Through the reversible process of albumins oxidation of its cysteine residues albumin is able to act as an antioxidant and can be used as a general oxidative biomarker for oxidative stress based diseases [Marrocco et al. 2017]. In this study albumin levels were shown to be in higher concentration in the low dose treatment group and lower in the high dose treatment group as compared to the control (Figure 2). The decrease in albumin in the high dose treated group is a change we would expect to see due to albumins antioxidant properties, but the higher concentration of albumin in the low dose treatment groups makes this evidence relatively inconclusive. In future study total protein

concentrations could be measured to demonstrate the relative albumin level compared to the total protein concentration. As expected from previous research antioxidant capacity in serum was lower in both treated groups as compared to the control (Figure 8). In the antioxidant assay kit used in this study it is assumed that the antioxidant activity is equivalent to the reducing capacity [Marrocco et al. 2017]. Therefore, lower antioxidant capacity demonstrates an inability of the organism to break down ROS, and correlates to an imbalance in the ROS to antioxidants ratio known as oxidative stress.

Another marker that can be used to demonstrate oxidative stress is gene expression of genes that code for antioxidants. The genes measured in this study are involved with the coding of proteins and enzymes that break down ROS. The genes analyzed in this study included Pdx1, Sod1, Gpx1, Prdx6, Ncf2, Duox1 and Ptgs2. The genes that showed noticeable increase in expression were Sod1 and Prdx6 (Figure 6). Sod1 is a gene that codes for the production of the enzyme copper-zinc superoxide dismutase, commonly called SOD1. SOD1 is a member of the SOD class of antioxidant enzymes responsible for detoxifying superoxide anions by converting them to hydrogen peroxide. Expression of Sod1 can be induced by redox-active metals and superoxides [Messner, Murray, & Kowdley 2012]. Mice with Sod1 knockout experienced increased liver tumors likely do increased ROS [Elchuri et al. 2005]. In our study Sod1 was upregulated in treated individuals likely do to an immune response to cellular damage from ROS. Prdx6 was also shown to be significantly upregulated in both of our treated groups. Prdx6 is a gene that codes for the protein PRDX6. PRDX6 has shown to be abundantly expressed in hepatocytes, implying that it counteracts circulating oxidants [Wang et al. 2003]. As with Sod1 this over expression of Prdx6 represents an immune

response to excessive ROS. The only gene that showed significant downregulation for both treated groups was *Ptgs2* (Figure 6). *Ptgs2* expression is known to increase in the presence of inflammatory stimuli [Hellman et al. 2015]. Thus, downregulation is not in line with our anticipated findings. We would expect inflammation to be present and triggering upregulation of the *Ptgs2* in treated individuals. Further study of the histology of hepatic tissue of 2AA treated groups could be carried out to assess the inflammation response to oxidative stress.

IgA protein concentration was found to be statically insignificant in treated groups as compared to the control (Figure 5) while AST activity was marginally increased in treated groups (Figure 3). IgA is an antibody produced in immune responses. Research has shown that the liver is very involved in the secretory IgA system, and liver disease has been shown to alter IgA metabolism [Brown & Kloppel 1989]. For this reason, we would have expected to see elevated IgA levels in the serum of treated individuals, though we were not able to demonstrate this relationship in this study. Elevated AST activity has been associated with liver damage in previous studies [Amin & Hamza 2005]. In this study we were only able to demonstrate slight elevation of the liver enzyme AST.

Conclusion and Future Direction

In conclusion some evidence of oxidative stress due to dietary exposure to 2AA was demonstrated in this study, but further study is needed to conclusively determine this effect. We were unable to conclusively demonstrate inflammation of hepatic tissues in this study. Further study to analyze the histology of liver tissues could be incorporated into future experiments to help demonstrate inflammation and damage to hepatic tissues.

Another approach to help quantify liver damage could be to incorporate quantification of serum alanine transaminase (ALT). ALT is a liver enzyme like AST that has been shown to be a very specific marker of hepatocellular injury [Hall & Cash 2012]. Additionally, body weight analysis could be performed in future animal study portions to help quantify toxicity of 2AA. This could also be coupled with a higher dose regimented treatment group to determine if toxicity was high enough to demonstrate adequate results in this study. Total protein analysis could also be incorporated into future experimental designs to quantify specific protein values as a relative concentration of the total protein concentration. This could help determine if elevated values represent an elevation in one specific protein or the total concentration as a whole.

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