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A Thesis

Entitled

The Role of Antioxidant Enzymes in the Induction of Phagocytic Activation by Dichloroacetate and Trichloroacetate Mixtures in Mice.

by

Kyle McIntosh

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Masters of Science Degree in Pharmaceutical Sciences

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The University of Toledo

May 2015

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An Abstract of

The Role of Antioxidant Enzymes in the Induction of Phagocytic Activation by Dichloroacetate and Trichloroacetate Mixtures in Mice.

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The University of Toledo

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The process of water chlorination results in production of different disinfection byproducts (DBPs), including dichloroacetate (DCA) and trichloroacetate (TCA). The compounds have been found to be hepatotoxic and hepatocarcinogenic in rodents. Previous studies have indicated the roles of oxidative stress (OS) and phagocytic activations in the induction of these effects in B6C3F1 mice. Also, previous studies have reported effects of DCA and TCA mixtures that ranged from additive to greater than additive on the induction of hepatic OS and additive to less than additive on the induction of phagocytic activation in mice. In this study, frozen peritoneal lavage cells collected from mice treated for those previous studies were used. In those studies, groups of mice were administered 7.5, 15, 30 mg/kg/day of DCA, 12.5, 25, 50 mg/kg/day of TCA, and 3 different mixtures of the compounds (Mix I, Mix II and Mix III) post orally for 13 weeks. The DCA: TCA ratios in Mix I, Mix II, Mix III corresponded to 7.5:12.5, 15:25, 30:50 mg/kg/day, respectively. Mice were then sacrificed and the peritoneal lavage cells (PLCs) were isolated and kept frozen at -80 C. Cells were assayed for the activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione

peroxidase (GSH-Px), as well as for the amount of glutathione (GSH). DCA, TCA and mixtures administration resulted in dose-dependent increases in SOD activity. Also, DCA, TCA, and mixture I treatments resulted in no change in CAT or GSH-Px activities while Mix II and Mix III resulted in significant increases in those enzyme activities. While 50 mg/kg/day TCA, and Mix I and Mix. II resulted in significant increases in total GSH levels; the rest of the other treatments did not result in significant changes in the levels of that biomarker. Failure of phagocytic activation has been previously suggested to contribute to increases in the hepatotoxic/ hepatocarcinogenic effects of DCA and TCA, and mixtures of high concentrations. The results of this study suggest that antioxidant enzymes and GSH play significant roles, in controlling the process of phagocytic activation, and that it significantly contributes to failure of this process to respond to high mixture concentrations.

Acknowledgements

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Chapter 1

Introduction

1.1Water Chlorination and Byproducts

Chlorine is a disinfectant that has become widely used in drinking water and swimming pools. Its use got started during epidemics of typhoid fever in the early 1900s, and since then typhoid fever mortality has dropped significantly [1]. Chlorine has been used in Canada and the United States due to its effectiveness at low concentrations and being a cost-effective treatment [2]. Other common disinfectants that can be used on drinking water are chloramine, ozone, and chlorine dioxide [3, 4]. Chlorine or these other disinfectants can react with natural organic material in source water resulting in a formation of disinfection byproducts (DBPs) [2, 5, 6, 7]. Conditions for forming these DBPs are the pH, temperature, chlorine concentration, chlorine reaction time, and character and amount of natural organic materials within the source water [6,7,8]. The classifications of these DBPs are placed into two groups: halogenated and non-halogenated compounds. One common class of DBPs is the haloacetic acids which includes dichloroacetic acid (DCA) and trichloroacetic acid (TCA) [3]. Mean concentrations of DCA detected in groundwater and surface water in the United States

have been 6.9 and 17 μg/litre, respectively [9], while TCA concentrations were 5.3 and 16 μg/litre, respectively [10].

There have been some concerns about the presence of these haloacetates in the drinking water. Animal toxicity studies in rats have shown certain DBPs can interfere with development and that mixtures of the disinfection products produce additive adverse effects [11]. DCA has been known to cause liver tumors in mice and rats, while TCA has been found to cause that in mice but not rats [12]. A case-control study reported a dose-response relationship between increase risk in brain cancer and duration of drinking chlorinated water among men [13], and another reported a relationship between long term exposure to chlorinated water and increase risk of bladder cancer in both, men and women [14]. The presence of DBPs, such as DCA and TCA in the drinking water, and a lifetime exposure of the public through consumption of chlorinated water have stimulated the interests of several investigators to study their long-term toxic effects.

1.2 Dichloroacetic acid (DCA)

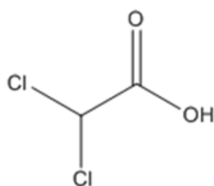


Figure 1: Chemical Structure of DCA

DCA has a pKa of 1.26 at 25°C, highly soluble in water as a salt [9]. It is used as a therapeutic agent for treatment of lactic acidosis, diabetes, and familial hyperlipidaemia. It is also used as a fungicide or chemical intermediate in manufacturing of pharmaceuticals, such as a topical astringent [9]. DCA is readily absorbed into the

bloodstream from the gastrointestinal tract. It is distributed to the liver, and muscles, and also to other organs such as spleen, brain, heart and kidney. The average half–life of DCA in the plasma of rats and humans are 2.97 and 0.43 hours, respectively [15]. The compound was believed to be metabolized in the liver generating glyoxylate, monochloroacetate, glycolate, oxalate, and carbon dioxide, and only 2% was found to be excreted in the urine of rats and mice as parent compound [16].

DCA has been shown to cause neurotoxic effects, developmental toxicity, and hepatocarcinogenicity in animal studies [11, 17, 18, 19]. Generally, these effects were seen when DCA was given at very high doses. The oral LD50 s of DCA in rats and mice were found to be 4.48 and 5.52 g/kg of body weight, respectively [9]. Moser et al. 1999 [18] reported sensitivity of F344 rats to the neurotoxic effects of DCA. Signs of DCA neuromuscular toxic effects included hind limb weakness, decrease motor activity and righting reflex, and a unique chest-clasping response. While exposure to high doses of DCA for 6 months resulted in permanent neurotoxic effects, administration of intermediate doses for 3 months or less were found to be slowly reversible [18]. Andrews et al. [11] indicated that 5000 µM of DCA can cause significant increase in rat embryo lethality and malformations. Signs of development toxicity included decreased head length, crown-rump length, and somite number. While several studies reported the carcinogenic effects of DCA [19,20,21,22,23], its genotoxicity remains controversial The World Health Organization (WHO) in 2000 [9] stated, "Although there are some evidences about DCA for being genotoxic, these effects occur at such high concentrations that they are not likely to be involved in the mode of DCA tumorigenesis." However, the United States Environmental Protection Agency (EPA) disagrees stating, "Because the

data on DCA genotoxicity *in vivo* are mixed, and because no clear explanation for the internal disagreement between studies is apparent, EPA considers it prudent to assume that DCA might be genotoxic, at least under *in vivo* exposure levels that are associated with detectable increases in tumor incidence". [9].

1.3 Trichloroacetic acid (TCA)

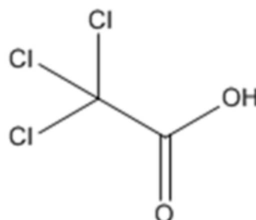


Figure 2: Chemical Structure of TCA

TCA has a pKa of 0.512-0.70 at 25oC [10]. The compound is used in soil steam sterilization and as a laboratory reagent in making variety of organic chemicals. Medically, TCA is used as a peeling agent and antiseptic. In industry, it is used as an etching and picking agent for metal surface treatments, and as solvent in plastic industry [10]. Like DCA, TCA is readily absorbed by the gastrointestinal tract, and then binds significantly to plasma proteins. A small portion of TCA was found to be metabolized mainly in the liver resulting in DCA and other metabolites. However, 48-65% of the compound was found to be excreted unchanged in the urine of mice, along with other metabolites [16].

TCA was found to cause liver and developmental toxicity, and to promote liver and kidney tumors [23, 24]. The oral LD50s of TCA in rats and mice were found to be 3.32 and 4.97 g/kg of body weight, respectively [10]. Long term studies with TCA in

mice showed increased risk of hepatocellular carcinomas [21, 25]. Smith et al. [26] have reported induction of developmental toxicity by the compound in Long-Evans rats, with fetuses developing significant reduction in body weight and length, as well as cardiovascular and renal malformations. Reports about the mutagenic potential of TCA are conflicting. TCA was reported as the least mutagenic of trichloroethylene metabolites and was found to be non-mutagenic in Samonella strain [27] there have been both positive and negative results towards TCA's ability to induce chromosomal damage *in vivo*. WHO has compiled data from various studies, and it was concluded that TCA possesses "neither significant mutagenic potential nor any structural alerts for mutagenicity" [10].

1.4 Oxidative stress (OS)

A free radical is a molecule with one or more unpaired electrons in its outer orbital. A molecule of oxygen has two unpaired electrons, but they are in different orbital, so it is not classified as a free radical. A molecule of oxygen can be converted to an oxygen free radical, or, can react with other radicals to form reactive oxygen species (ROS). Molecular oxygen undergoes reduction into water in the mitochondria (shown in Figure 3); generating oxygen free radicals or reactive oxygen species (ROS) as intermediates. These ROS include, superoxide anion ($\bullet O_2^-$) (SA), the relatively stable hydrogen peroxide (H_2O_2), and most reactive hydroxyl radical (•OH) [28]. When there is an interruption in the reduction of oxygen to water; levels of ROS dramatically increase causing oxidative stress (OS).

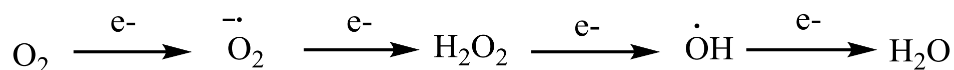
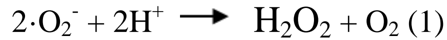


Figure 3: Reduction of O_2 to H_2O.

The term OS refers to the imbalance between prooxidant and antioxidant levels, where the reaction is shifted in favor of prooxidants production in cells and tissues [29], causing oxidative tissue damage. The damage can involve modifications of lipids, proteins, and DNA molecules of cells, leading to the development of various pathological conditions, such as cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, rheumatoid arthritis, ageing, Alzheimer's and Parkinson's disease [30]. OS may also result from exposure to xenobiotics that can be converted to free radicals *in vivo*, leading to generation of ROS through the process of redox cycling [31], or through the process of phagocytic activation [32]. Other causes for production of OS include, low dietary antioxidants, heavy exercise, or mutations occurring to antioxidants [29, 33].

Organisms use antioxidant defenses, including antioxidant enzymes to protect themselves against ROS. These enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-px). Their reactions are shown in equations 1-3, below. Antioxidant defenses also include non enzymatic molecules, such as natural antioxidants that are found in foods or supplements such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E), and the endogenous antioxidant molecule, glutathione (GSH) [30].

SOD catalyzes the dismutation of SA into oxygen and hydrogen peroxide (H_2O_2), as shown in equation (1) [34]. The enzyme utilizes zinc and copper as cofactors.



$$2 \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

CAT is found in the peroxisomal/glyoxysomal system. The enzyme converts H_2O_2 generated from SOD's catalyzation or from synthesis in the peroxisomes into water and oxygen molecules, as shown in equation (2) [34].

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

This enzyme is important when H_2O_2 (use subscripts) levels are low. When hydrogen peroxide concentrations are high, GSH-px contributes to its detoxification [29].

GSH-px is found in the cytoplasm of mammalian tissues. Using reduced glutathione (GSH) as a cofactor, it inactivates H_2O_2 and other peroxides, as shown in the equation (3) [34]. The oxidized form of glutathione is glutathione disulphide (GSSG). "Glutathione is a major soluble antioxidant that is highly abundant in the cytosol (1-11 mM), nuclei (3-15 mM), and mitochondria (5-11 mM) [30].

$$2\ GSH_{(reduced)} + H_2O_2 \rightarrow GSSG_{(oxidized)} + 2H_2O \quad (3)$$

1.5 Phagocytic activation

When a site becomes injured from environmental pathogens, macrophages, neutrophils and other phagocytes become activated, migrate to the site, and generate a massive amount of ROS to destroy the pathogens. This massive production of ROS in an inflammatory environment is called respiratory burst, or oxidative burst [28, 31]. The oxidants in phagocytic cells are produced by the actions of enzymes, including NADPH oxidase, nitric oxide synthase, and myeloperoxidase that are responsible for the production of SA, nitric oxide (NO), and H_2O_2, respectively. Although these oxidants are supposed to exert antimicrobial activity, they can damage healthy tissues and cells

adjacent to the injured site [28]. While phagocytic activation has important protective roles against several infectious diseases and cancer, it can be damaging especially after exposure to xenobiotics that can produce a primary tissue injury, resulting in migration of phagocytic cells to the site of injury and producing more tissue damage.

1.6 Oxidative Stress and DCA and TCA

The induction of hepatotoxicity by DCA- and TCA in mice was found to be associated with the production of OS after acute and long-term exposure [16, 19, 35]. Biomarkers of hepatic OS that were found to be induced in response to the compounds included production of SA [36, 37], lipid peroxidation (LP) [16, 36], DNA damage [20, 22, 36, 38], and changes in antioxidant enzyme activities [39]. These biomarkers were also found to be induced in the hepatic tissues of mice exposed to mixtures of DCA and TCA [40].

1.7 Phagocytic activation and DCA and TCA

Previous *in vitro* studies in J774A.1 macrophages reported concentration-dependent increases in SA production in response to DCA and TCA, which were associated with concentration-dependent increases in cellular death at different time points [41]. Other studies on J774A.1macrophages have examined the protective roles of polyclonal tumor necrosis factor-alpha (TNF-α) [42], as well as the roles of antioxidant enzymes [43], against the induction of SA and cell death by the compounds. The role of phagocytic activation in DCA and TCA toxicities had been also studied *in vivo* in mice. Acute exposure of mice to high doses of DCA and TCA was found to induce phagocytic activation of peritoneal lavage cells PLC, as indicated by SA production by these cells [36]. Various biomarkers of phagocytic activation, including SA and TNF-alpha

production and increases in myeloperoxidase activity were also found to be induced in dose- and time-dependent manners in PLC of mice, after long term exposure to DCA and TCA [44, 45]. Phagocytic activation was also found to be induced in PLC of mice exposed to mixtures of DCA and TCA. Recent studies on different mixtures of DCA and TCA administered to mice subchronically, indicated production of effects that ranged from additive to less than additive on the induction of various biomarkers of phagocytic activation in PLC, with increasing the mixture concentrations [46].

Chapter 2

Objectives

DCA and TCA are byproducts produced during the process of drinking water chlorination. Previous studies have indicated induction of phagocytic activation by DCA and TCA in the PLCs of B6C3F1 mice [44, 45]. Phagocytic activation in PLCs of mice has been also shown to be induced in response to mixtures of DCA and TCA with net effects changed from additive to less than additive with increasing mixture concentrations and was suggested to play a role in the compounds- and mixtures-induced hepatotoxicity and hepatocarcinogenicity [46]. However, studies on the effects of DCA and TCA mixtures in hepatic tissues of mice have reported net effects of additive that changed to greater than additive with increasing concentrations [46], and that antioxidant enzymes play an important role in the observed changes of mixture effects. Since the role of antioxidant enzymes in phagocytic activation has been established [45], we proposed that these enzymes, as well as glutathione play roles in modulating the mixtures effects. In this study, activities of the antioxidant enzymes SOD, CAT and GSH-PX, as well as the amounts of glutathione were determined in frozen PLCs that had been obtained originally

from mice treated subchronically with different doses of DCA, TCA and three mixtures of the compounds for the previously mentioned studies.

Chapter 3

Materials and Methods

3.1 Chemicals

The chemicals used for these studies were purchased from Sigma Aldrich (St. Louis, MO), at the highest grade available.

3.2 Animals and Treatments

The study did not involve using live animals. However, frozen peritoneal lavage cells collected from animals previously treated for studies related to the current one were used. The cells were originally obtained from male B6C3F1 mice that were purchased from Harlan Teklad (Indianapolis, IN) at 6 weeks of age. The animals weighed about 20 grams and were allowed to adapt to their environment for 3 days before they were treated. The animals were housed in temperature-controlled rooms with a 12-hour light/dark cycle and were given free access to food and water. The mice were divided into 10 groups (6 animals each), that were assigned to a control group and 9 treatment groups. Animals in the control group received distilled water (pH adjusted to 7.0 with NaOH solution), via oral gavage, daily for 13 weeks at a volume of 5 ml/kg body weight. Three treatment groups were given DCA at doses of 7.5, 15 and 30 mg/kg/day, while another 3 treatment groups were given TCA at doses of 12.5, 25, and 50 mg/kg/day. The

remaining 3 treatment groups received different mixtures (Mix I, II, and III) of DCA and TCA. The DCA: TCA ratios in Mix I, II, and III were correlated to 7.5:12.5, 15:25, and 30:50 mg/kg/day, respectively. The compounds were dissolved in distilled water (pH of each solution was adjusted to 7.0 with NaOH solution). At the end of the treatment period, the mice were euthanized with carbon dioxide anesthesia followed by cervical dislocation, and peritoneal lavage cells (PLCs) were collected. The release of PLCs from the peritoneal cavity was done by injecting 3 ml of buffer into the cavity, followed by massaging the abdomen for 30 seconds for each mouse. The buffer was consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, and 2 mM CaCl2 (pH 7.3). Fluid was then withdrawn from the peritoneal cavity by syringe and was centrifuged at 1700 × g for 10 minutes. After centrifugation, supernatant was collected from each sample and discarded. PLC pellets were re-suspended in 2 ml of Dulbecco's Modified Eagle's Medium containing L-methionine 200 mM L-glutamine, penicillin-streptomycin solution (10,000 U penicillin + 10,00 μg streptomycin)/ ml, 1 M HEPES buffer, 10 mM minimum essential medium-nonessential amino acids (MEM-NEAA), 100 mM sodium pyruvate solution, and 10% fetal bovine serum. The viability of the PLCs was confirmed under the microscope, using the trypan blue exclusion method. All cells in different treatment groups and the control were found to be 98-99% viable. The PLCs suspensions were stored in tubes at a freezer at -80º C, and they were used later for the determination of superoxide anion (SOD), Catalase (CAT), glutathione peroxidase (GSH-PX) and glutathione (GSH), as indicated below.

3.3 Determination of Superoxide Dismutase (SOD) Activity

SOD activity was determined in the PLCs, according to the method of Marklund and Marklund [47], with modifications. The method is based on measuring the percentage inhibition of pyrogallol autooxidation by SOD. The reaction mixtures contained 200 µl of cell suspension, 750 µl of Tris-cacodylic buffer and 250 µl of 2 mM pyrogallol. Tris-cacodylic buffer was made up of 50 mM Tris-HCL, 50 mM cacodylic acid and 1mM EDTA-pentasodium acetic acid (pH 8.2). The reaction mixtures were left for 20 seconds at room temperature before taking the first absorbance reading at 420 nm. After first reading, absorbances were recorded every 30 seconds over a period of 3 minutes, using a Genesys™ 20 Spectrophotometer (Thermo Fisher Scientific, Madison, WI). A unit of SOD is defined as the amount of SOD required to produce 50% inhibition of pyrogallol auto-oxidation. The number of SOD units was divided by the amount of protein in each sample.

3.4 Determination of catalase (CAT) activity

Catalase activity in the PLCs was determined according to the method of Cohen et al. [48], with modifications. The method was based on the decomposition of hydrogen peroxide by CAT in the presence of potassium permanganate. The assay required three reactions to be run in three tubes labeled as sample, blank and standard tubes. The sample tube contained 100 µl of cellular suspension, the blank tube contained 100 µl of sucrose buffer, and the standard tube contained 1.1 ml of deionized water. One milliliter of 6 mM H_2O_2 solution was added to each of the sample and the blank tubes. The sample and blank were vortexed and the three reaction tubes were then incubated on ice for 3 minutes and reaction were stopped by adding 200 µl of 6 N H_2SO_4 to each tube. A total of 1.4 ml

of 0.002M $KMnO_4$ was added to each tube and the mixtures were quickly vortexed for (3-5 seconds). Absorbances were recorded at 480nm immediately, to avoid precipitation of reactants, using a Genesys™ 20 Spectrophotometer (Thermo Fisher Scientific, Madison, WI).

One unit of enzyme activity equals to k/ (0.00693 where:

$$k = \log [(S0/S1) \times 2.3/t]$$

S0 = absorbance of standard minus the absorbance of blank

S1 = absorbance of standard minus the absorbance of sample

t = time of incubation (3 minutes).

The number of CAT units was divided by the amount of protein in each sample.

3.5 Determination of glutathione peroxidase (GSH-Px) activity

GSH-Px activity was determined in the PLCs using the method of Lawrence and Burk [49], with modifications. The method is based on the oxidation of reduced glutathione by glutathione peroxidase and the regeneration of reduced glutathione utilizing glutathione reductase and NADPH.

The reaction tubes contained 100 µl of cellular suspension mixed with 700 µl of a reaction mixture and 200 µl of glutathione disulfide (GSSG) reductase. The reaction mixture contained 1mM EDTA, 1 mM NaN3, 0.2 mM NADPH and 1 mM GSH in Phosphate Buffer Saline (PBS). The tubes were vortexed 20-30 seconds and 100 µl of H_2O_2 (0.25 mM, final concentration) was added to each tube. Absorbances were immediately recorded after that and then every 30 seconds for 3 minutes at 340 nm, using a Genesys™ 20 Spectrophotometer (Thermo Fisher Scientific, Madison, WI).

Absorbances were converted to nanomoles of NADPH using an extinction coefficient of 6.22×10^3 L $mol^{-1}cm^{-1}$ [49]. Enzyme activity was reported as nanomoles of NADPH oxidized per minute per mg protein.

3.6 Determination of Glutathione (GSH)

Total GSH (GSH-reduced + GSSG-oxidized) was determined by the recycling assay described by Anderson [50] with modifications. The assay was based on GSH oxidation by 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) to form 5-thio-2 nitrobenzoic acid (TNB), that is measureable at 412 nm. The GSSG formed during the reaction was recycled to GSH by the action of GSSG reductase in the presence of NADPH.

The assay required a stock buffer containing 143 mM dibasic sodium phosphate, and 6.3 mM tetrasodium EDTA, pH 7.5, the daily buffer containing 0.248 mg/ml NADPH in stock buffer, and GSSG reductase solution containing 266 units of the enzyme per milliliter of stock buffer. The assay mixture contained 25 µl of cellular suspension, 700 µl daily buffer, 100 µl DTNB solution, and 175 µl deionized water. Ten microliters of GSSG-reductase solution was added to the mixtures and absorbances were immediately recorded, and then every 30 seconds for 3 minutes, using a Genesys™ 20 Spectrophotometer (Thermo Fisher Scientific, Madison, WI). A standard curve for GSH was prepared following aforementioned method, but replacing the 25 µl of samples with 25 µl standard solutions containing 1-4 nmole of GSH. Results of the standards are shown in figure 4.

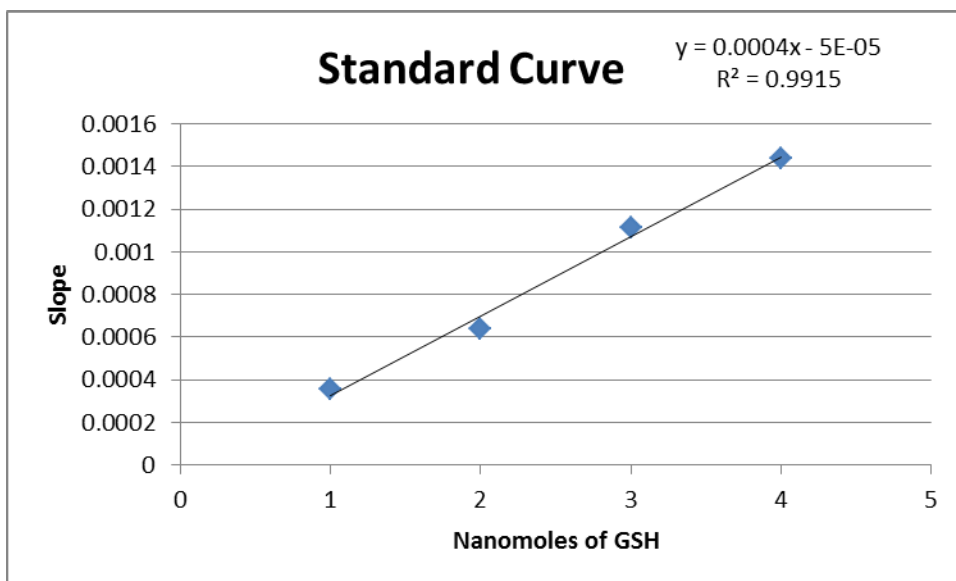


Figure 4: Glutathione Standard Curve

3.7 Determination of Protein

Protein concentrations were determined according to the method of Lowry et al. [51] using bovine serum albumin (BSA) as a standard. Results of the BSA standards are shown in figure 5.

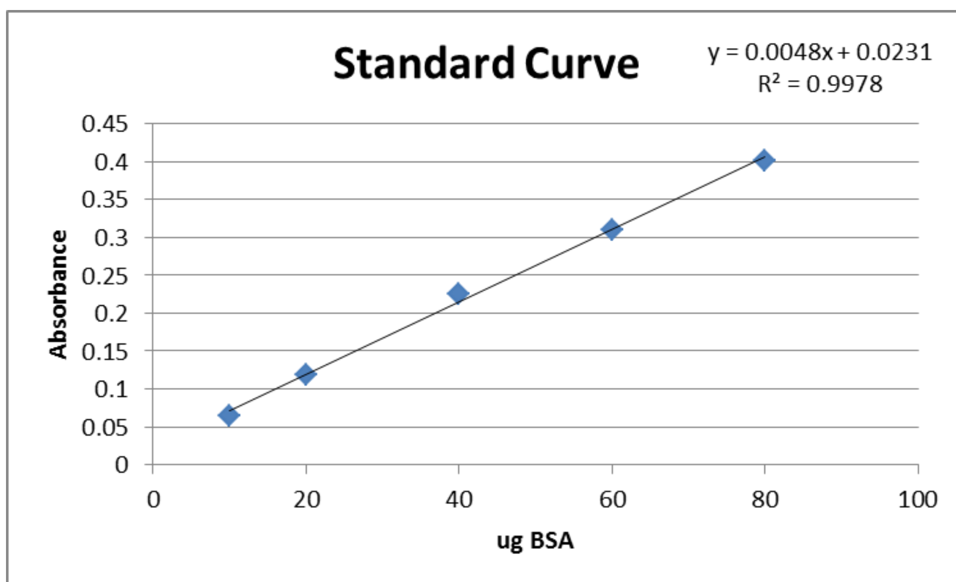


Figure 5: BSA Standard Curve

3.8 Statistical Methods

Data were analyzed using the Microsoft Excel data analysis tool package and IBM SPSS Statistics Data Editor. All data were expressed as means of six samples (animals) ± SD. A one-way analysis of variance (ANOVA) was used to determine statistical difference between groups, with Fisher's Least Significant Difference (LSD) method used as a post hoc test. A significance level of $p < 0.05$ was used in all of the statistical analyses. Pearson's correlation coefficients were determined to assess correlations between the studied antioxidant enzyme activities and GSH.

Chapter 4

Results

The effects of various doses of DCA, TCA and mixtures of the compounds (Mix I, Mix II, Mix III) on SOD activity of PLCs of mice are shown in figure 6. Individual treatment with DCA and TCA resulted in significant and dose-dependent increases in SOD activity, when compared with the control, and also when the responses to various doses of each of the compounds were compared with each other. While the figure also shows significant increases in SOD activity in response to the three mixtures when compared with the control, the increases induced by Mix II and III were not significantly different. Figure 6 also shows that the increase in SOD activity induced by Mix II and III were significantly higher than all of the increases induced by the individual compounds and by Mix I.

Figure 7 illustrates changes in CAT activity of the PLC's of mice, 13 weeks after administration of different doses of DCA, TCA and three different mixtures of the compounds. While no significant effects on the enzyme activity were induced by individual doses of DCA or TCA, and by Mix I, significant inductions of that enzyme activity was observed with Mix II and III, as compared with control. The figure also shows that Mix III-induced CAT activity was significantly greater than that induced by Mix II.

The effects of treatments of mice with different doses of DCA, TCA, and three different mixtures on GSH-Px activity of PLCs are shown in figure 8. Similar to the effects on CAT activity, GSH-Px activity was not changed in response to any of the individual concentrations of DCA and TCA and also Mix I when compared with the control. However, significant increases in GSH-Px activity were observed with Mix II and III when compared with the control. Also, Mix III-induced increase in GSH-Px activity was significantly greater than that induced by Mix II.

Figure 9 illustrates the effects of treatment of mice with different doses of DCA and TCA, and also with three different mixtures on total glutathione (GSH) levels in PLCs. Except for treatment with the highest dose of TCA that resulted in significant increase in GSH level, none of the other individual doses of TCA or DCA resulted in significant changes in the level of that biomarker, when compared to the control. The figure also shows that Mix I did not result in a significant change in GSH level, but Mix II and III resulted in significant increases in the levels of that biomarker when compared with the control. Also, the increase in GSH level in response to Mix III was significantly greater than that induced by Mix II.

Pearson's correlation coefficients were calculated to assess the correlations between changes in various antioxidant enzyme activities and GSH in response to treatments with DCA, TCA and the mixtures (table 1). A Pearson's correlation coefficient approximating 1.0 indicates a strong correlation between any two tested responses. The table shows good correlations between those biomarkers in response to treatment with mixtures. The calculations also revealed weak correlations between the antioxidant markers in response to DCA and TCA treatments.

Chapter 5

Discussion

Previous studies indicated induction of phagocytic activation of PLCs of mice by similar DCA and TCA doses and mixture concentrations used for this study, with SA used as one of the markers to assess induction of that mechanism [46]. The studies showed that DCA, TCA induced dose-dependent increases in that biomarker, and that the mixtures also induced significant increases in that biomarker, but mixture III induced a similar increase in SA level as that induced by Mix II. [46]. Since phagocytic activation was found to play a role in DCA- and TCA-induced hepatic OS that is associated with liver toxicity and carcinogenicity in mice [19, 21, 22, 25, 36], and that antioxidant enzymes and glutathione play roles in the induction of various biomarkers of OS in response to the compounds and their mixtures [39, 40], it is important to assess the contribution of these antioxidant mechanisms to the observed changes in SA levels in the PLCs, in response to various treatments.

SA is known to undergo dismutation by the action of SOD, which results in H_2O_2 overproduction [29, 34]. However H_2O_2 is a more oxidizing ROS than SA, but can be converted to H_2O by the actions of CAT and GSH-Px [28, 29, 33]. The observed dose-

dependent increases in SOD activity in response to DCA, TCA and the mixtures indicate that SA generated in PLCs underwent conversion to H_2O_2. Since the increases in SOD activity in response to mixture III is greater than that of mixtures II, and previous studies showed similar levels of SA induction by the two mixtures [46], it is concluded that SOD induction by mixture III was sufficient to bring the SA level to that induced by Mix II. Hence H_2O_2 level in response to Mix III is expected to be higher than that produced in response to Mix II. Phagocytic activation was suggested to play a role in the protection against DCA- and TCA-induced liver toxicity and cancer in mice [36, 44]. Therefore the leveling of phagocytic activation in response to Mix III may indicate no further protection provided by this mechanism against possible hepatotoxic/ hepatocarcinogenic effects of mixture concentrations higher than those in Mix II. While the dose-dependent increases in SOD activity is expected to be associated with production of higher and dose-dependent increases in H_2O_2 levels, the no changes in CAT and GSH-Px activities in response to DCA and TCA indicate accumulation of this more damaging species in the PLC. This can be also confirmed by the weak correlation coefficient obtained for SOD-CAT, and SOD-GSH-px in response to the two compounds (table 1). However, H_2O_2 is found to play a role in the induction of myeloperoxidase (MPO) activity [52, 53, 54] which is considered another biomarker of phagocytic activation [53, 54]. Therefore accumulation of H_2O_2 could have contributed to the induction of MPO, which may have further contributed to the increases in phagocytic activation in response to DCA and TCA. This may also suggest contribution of that mechanism to the protection against liver toxicity induced by these doses of the compound. The latter suggestion may be confirmed by previous studies indicating no observed liver toxicity by DCA and TCA

doses falling in the range of those used for this study [39, 40]. However, the increases in CAT and GSH-Px activities in response to the mixtures suggest possible contribution of MPO through H_2O_2 production. This may also confirm previous finding about production of dose –dependent increases in MPO activity in PLC of mice, in response to the same doses of DCA and TCA and Mix II and III, used in this study [44, 45]. Although the results suggest protection failure by this mechanism against liver toxicity and cancer in response to Mix III, i.e., potentials of mixtures with concentrations higher than those of Mix II to induce liver toxicity/ cancer, further histopathological studies are required to assess that. GSH is an antioxidant molecule that is a known substrate for GSH-Px and can also protect against production of ROS and free radicals [55, 56, 57]. While the no change in GSH level in response to DCA and TCA doses may have contributed to the no change in GSH-Px activity, the observed increases in GSH level in response to Mix. II and III have contributed to the increases in the activity of that enzyme in response to these mixtures. This can be also confirmed by the good correlation between the two biomarkers in response to the mixtures (table 1). The reason for the increases in the GSH levels in response to Mix II and III cannot be explained here, but future assessment of GSH S-transferase activity is needed, since activity of this enzyme was found to be modulated by at least DCA [58, 59]. The increase in GSH that was not associated with increase in GSH-Px activity in response to the highest dose of TCA may indicate involvement of GSH in other function than GSH-Px. GSH can protect against production of free radicals, besides ROS [57]. It is possible that GSH was involved in neutralizing free radicals that are generated by the process of metabolism. However, further studies are required to assess the nature of free radicals that may be generated in the PLCs.

In summary, antioxidant enzymes and GSH are found to play a role in the induction of phagocytic activation by DCA, TCA and mixtures of the compounds by modulating the levels of different ROS in PLCs. Since phagocytic activation was indicated to play a role in the protection against DCA- and TCA-induced hepatotoxicity and cancer, it is suggested that changes in SOD activities with no changes in other enzyme activities or GSH can contribute to the protection by this mechanism against production of liver toxicity/ cancer by DCA and TCA doses used in this study. However, changes in SOD activities associated with changes in other antioxidant enzyme activities and GSH may contribute to failure of this mechanism to provide protection against certain concentrations of the compounds mixtures.

Chapter 6

Recommendations for Future Studies

1. Since GSH catalyzes not only GSH-Px but also glutathione S-transferase future investigations on the activity of glutathione S-transerfase in response to DCA and TCA mixtures are needed.

2. Due to increase in antioxidant activity with Mix II and Mix III, phagocytic activation may fail to protect against liver mixtures-induced liver cancer. i.e., the controlling action of the antioxidant mechanisms in the phagocytes limits the protective action of this mechanism against mixture-induced cancer. This is especially important since biomarkers of hepatic oxidative tissue damage, including LP, were found to undergo additive and greater than additive increases in response to Mix II and III, respectively [40]. Future histopathological studies on liver tissues are needed to determine that possibility.

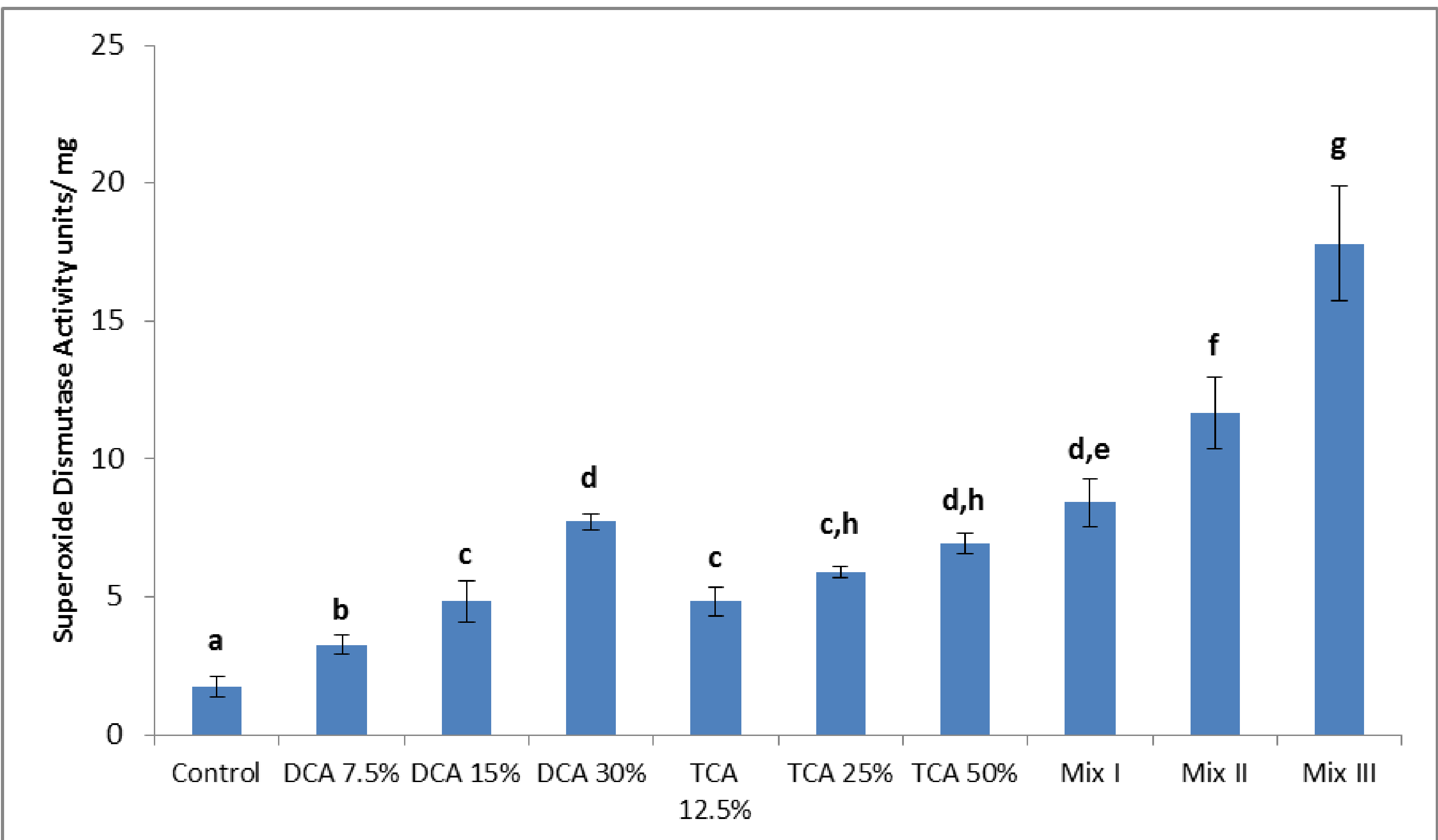


Figure (6): SOD activity of PLCs from control mice and mice treated with DCA, TCA, and mixtures of the compounds for 13 weeks. Columns that do not share an identical superscript are significantly different.

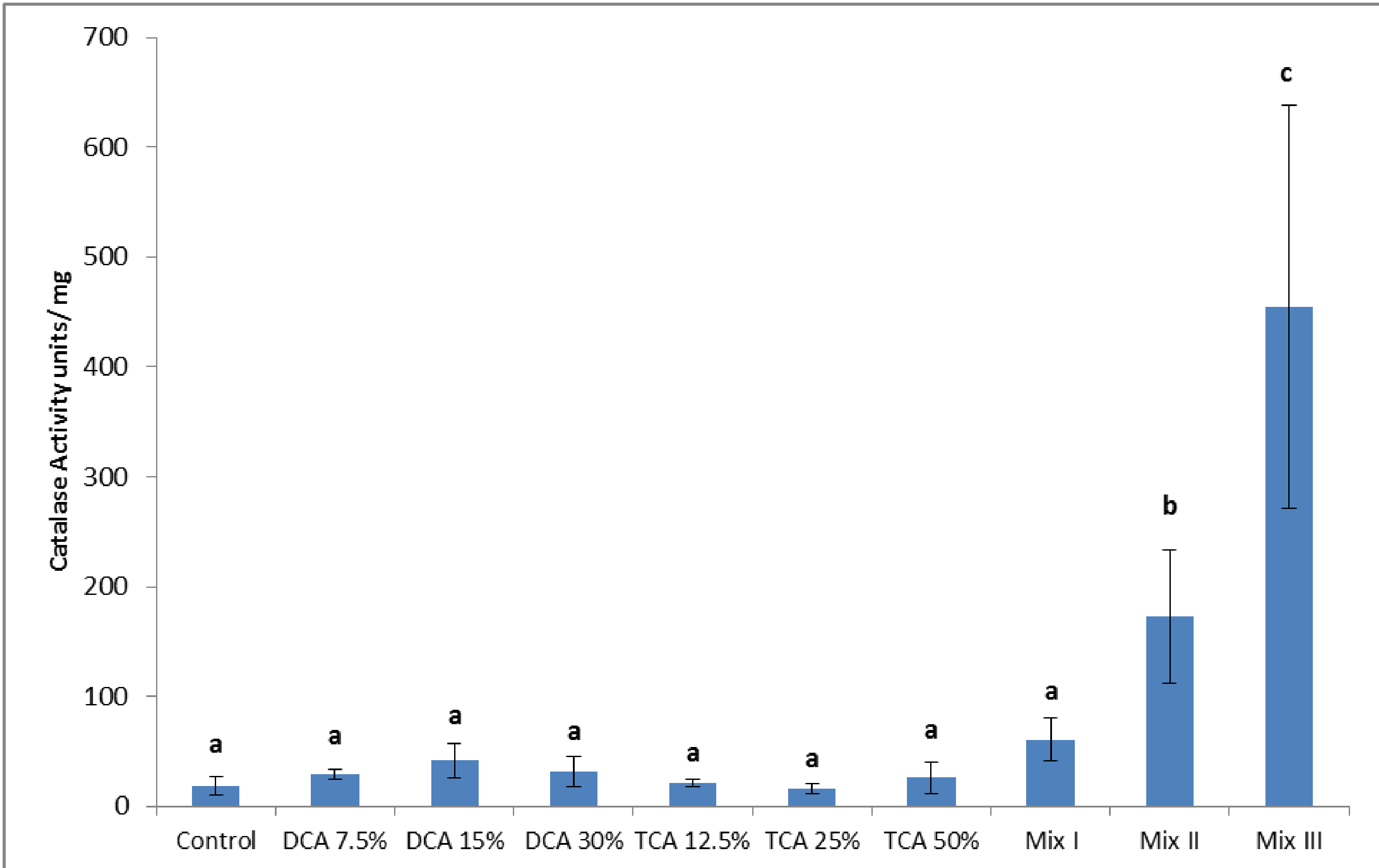


Figure (7): CAT activity of PLCs from control mice and mice treated with DCA, TCA, and mixtures of the compounds for 13 weeks. Columns with unidentical superscripts are significantly different, using single factor ANOVA with $p<0.05$.

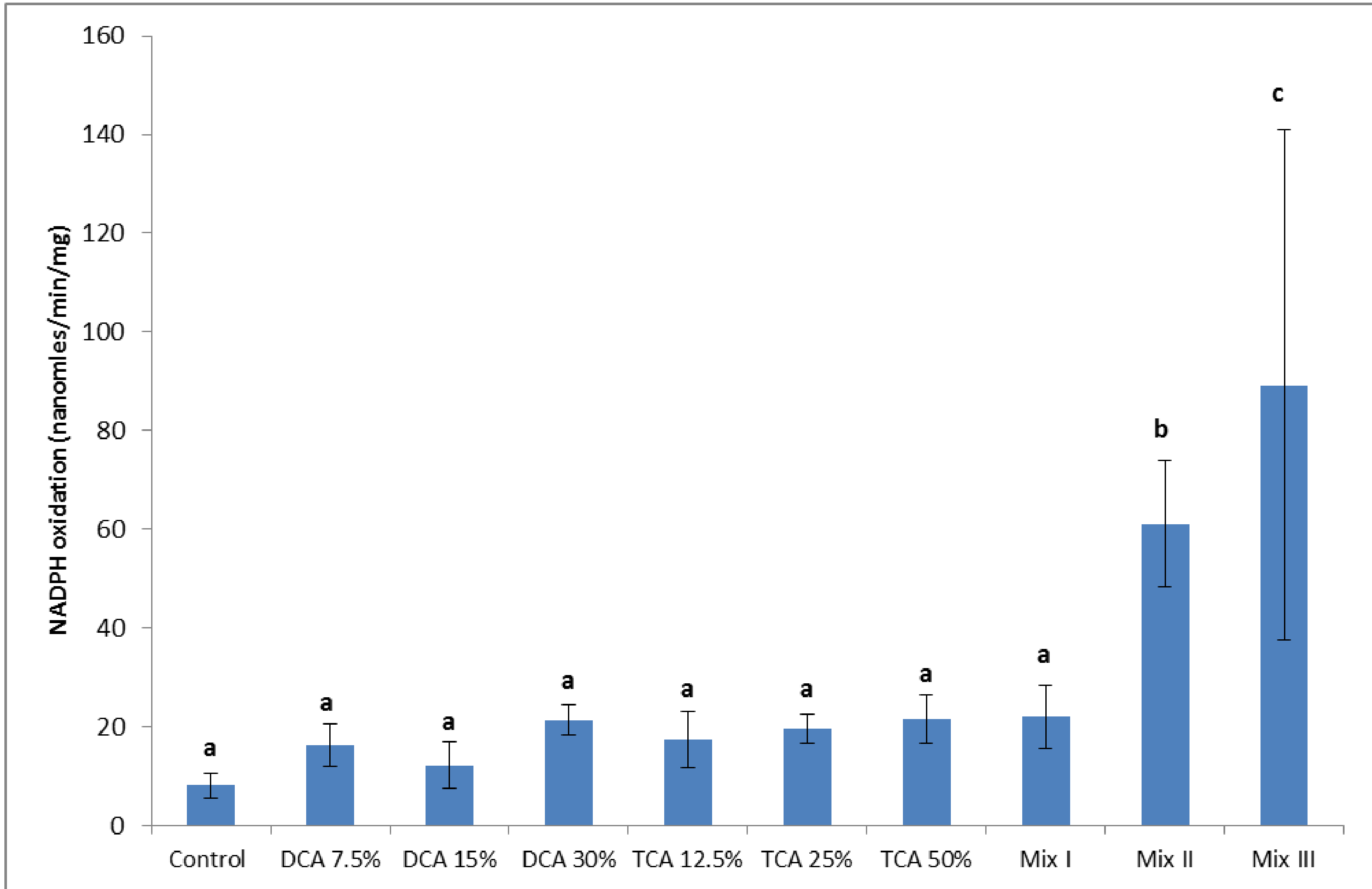


Figure (8): NADPH oxidation by GSH-Px activity of PLCs from control mice and mice treated with DCA, TCA, and mixtures of the compounds for 13 weeks. Columns with unidentical superscripts are significantly different, using single factor ANOVA with $p<0.05$.

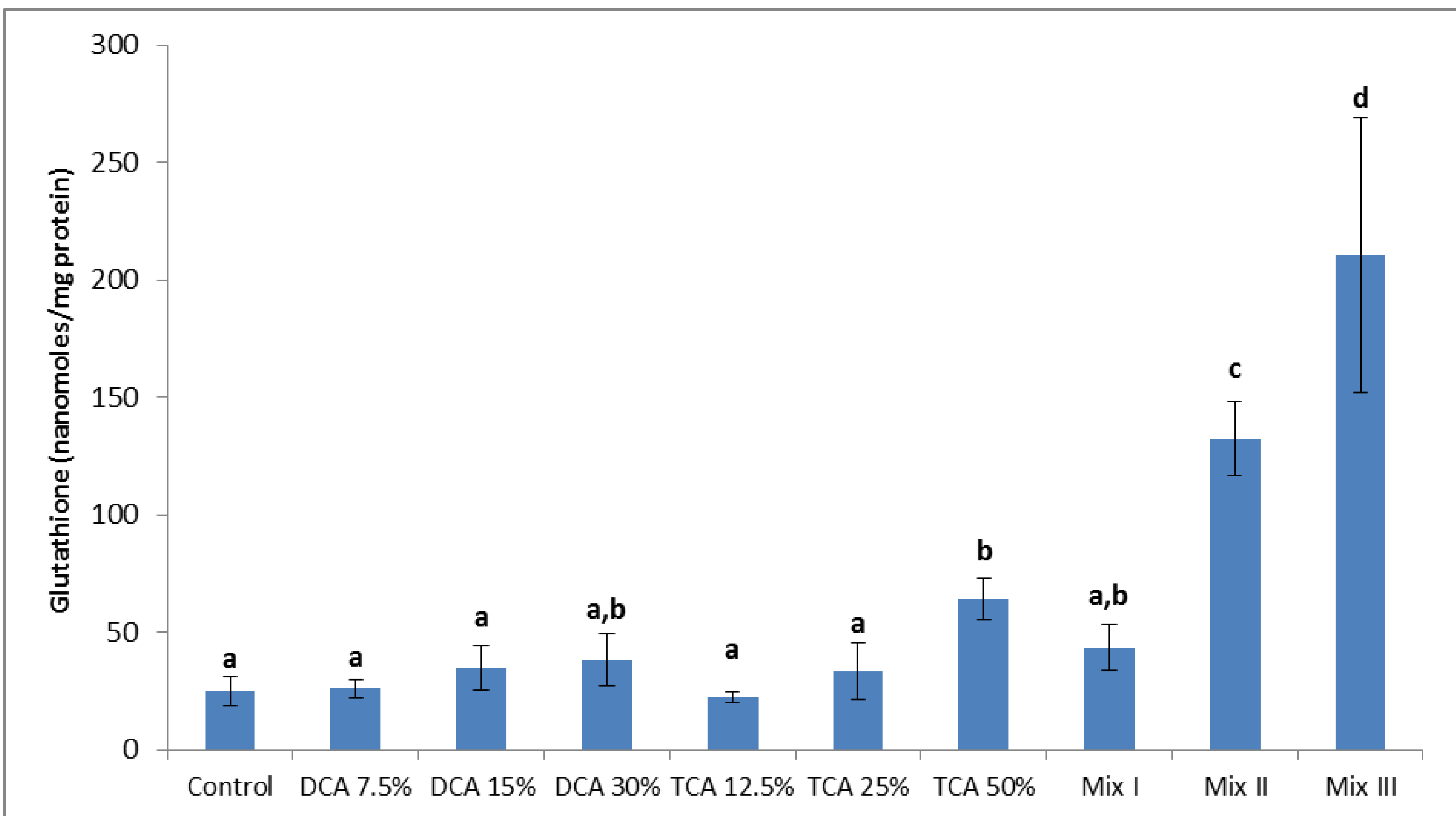


Figure (9): Total GSH determined in PLCs of control mice and mice treated with DCA, TCA, and mixtures of the compounds for 13 weeks. Columns that do not share an identical superscript are significantly different.

Table 1: Correlations between activities of enzymes involved in response to treatments with DCA, TCA and mixtures of the compounds. Data for one enzyme in response to all doses of each treatment (DCA, TCA and Mixtures), were pooled and compared with pooled data for the other enzyme or GSH in response to all of the doses of the same treatment.

Pearson's Correlation Coefficient			
	DCA	TCA	Mixtures
SOD Activity vs. CAT Activity	-0.06505	0.03598	0.71016
SOD Activity vs. GSH-Px Activity	0.43972	0.27314	0.52465
Total GSH Levels vs. GSH-Px Activity	-0.04481	0.10014	0.66609

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