

2011

Studies on the long term effects of marginal vitamin E deficiency on dichloroacetate- and trichloroacetate- induced phagocytic activation in mice

Ali Al-Dieri
The University of Toledo

Follow this and additional works at: <http://utdr.utoledo.edu/theses-dissertations>

Recommended Citation

Al-Dieri, Ali, "Studies on the long term effects of marginal vitamin E deficiency on dichloroacetate- and trichloroacetate- induced phagocytic activation in mice" (2011). *Theses and Dissertations*. 505.
<http://utdr.utoledo.edu/theses-dissertations/505>

This Thesis is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's [About page](#).

A Thesis

entitled

Studies on the Long Term Effects of Marginal Vitamin E Deficiency on Dichloroacetate-
and Trichloroacetate- Induced Phagocytic Activation in Mice

by

Ali Al-Dieri

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

Dr. Ezdihar Hassoun, Committee Chair

Dr. Zahoor Shah, Committee Member

Dr. Ming-Cheh Liu, Committee Member

Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

May 2011

Copyright 2011, Ali Al-Dieri

This document is copyrighted material. Under copyright law, no parts of this document may be reproduced without the expressed permission of the author.

An Abstract of
Studies on the Long Term Effects of Marginal Vitamin E Deficiency on Dichloroacetate-
and Trichloroacetate- Induced Phagocytic Activation in Mice

by

Ali Al-Dieri

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

The University of Toledo

May 2011

Dichloroacetate (DCA) and trichloroacetate (TCA) are major byproducts of water chlorination, and are also formed, *in vivo* by the metabolism of trichloroethylene (TCE), a common water contaminant. Previous studies in our lab have shown that subchronic exposure to both DCA and TCA induces phagocytic activation in the peritoneal lavage cells (PLCs) of mice. In this study, we assessed the effects of marginal vitamin E deficiency on phagocytic activation induced by DCA and TCA in B6C3F1 mice. Animals were divided in two groups, where one was kept on a regular diet supplemented with vitamin E (E-normal) and the other kept on a low vitamin E diet (E-deficient group). The two groups were divided into subgroups and were treated everyday, by gavage, with distilled water as the control, and 77mg/kg/day of DCA or TCA for a period of 13 weeks.

The peritoneal lavage cells (PLCs) were collected from the animals after they were euthanized and different biomarkers of phagocytic activation, including superoxide anion (SA) and tumor necrosis factor alpha (TNF- α), as well as the activity of myeloperoxidase (MPO) were determined. Antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were also assessed. Significant increases in the different biomarkers in response to DCA and TCA were reported in the E-normal and E-deficient diet groups when compared to the corresponding controls in each group. However, the increases in response to the compounds in the E-deficient groups were significantly greater than those observed in the E-normal group. Since previous studies have suggested the contribution of phagocytic activation to the protection against DCA and TCA long term effects, the increases in the biomarker of phagocytic activation in the E-deficient groups may indicate further protection provided by this mechanism against the effects induced by these compounds. SOD activity was also significantly increased in response to the compounds in either group, with more significant increases observed in the E-deficient group as compared with the E-normal group. CAT and GSH-Px activities on the other hand did not change in response to the compounds in the E-normal group but were significantly increased in the E-deficient group when compared with the corresponding controls. The increases in the antioxidant enzyme activities in response to the compounds in the E-deficient groups indicate significant SA dismutation, and conversion to water. They also suggest up-regulation of the enzyme activities in response to vitamin E deficiency, accommodating for the loss of protection by vitamin E against the compounds long term effects.

Acknowledgements

This research project would not have been possible without the support of many people. I would like to take the opportunity and give thanks to everyone here at the University of Toledo for their help and support throughout my many years here. It has been a wonderful and rewarding journey.

First and foremost, I want to express my deepest and utmost gratitude to my advisor, Dr. Hassoun. This thesis would not have been possible without the guidance and support of Dr. Hassoun. It has been an honor to work under someone who is competent not only at the research and academic level, but also an expert and respected person in her field. I am forever grateful for everything she has done for me.

I also wish to thank my two other committee members, Dr. Zahoor Shah and Dr. Ming-Cheh Liu for their support, guidance, and contribution in the aid of preparing my thesis. It has been an honor for me to have them.

I would like to thank Dr. Surya Nauli for his support and assistance in sparking my interest in research during my undergraduate year. I will always be appreciative for the opportunity he granted me to be in his lab. Thank you.

I thank Jacquelyn Cearfoss for guiding me in the lab and with the animals every time I needed help and helping me master almost every biochemical technique I did in my research.

I would also like to give thanks to my fellow graduate students for being there for me and each other from day one. Thank you Chris, Adnan, Amanda, and Yasir. Special thanks go to my best friends Yasir and Adnan, their support and encouragement was constantly available not only during my research and academic life but also during my personal life.

Last but not the least; I would like to express my never ending love and gratitude to my beloved family and the Almighty God. My best friend, soul mate, and wife, her love, support and patience was an inspiration during this research experience. My two wonderful sons, Hussein and Abbas. Thank you mother and father for your understanding and endless love, through the duration of my studies. Thank you for being proud of me. You express that often and it means a great deal to me. Thank you, both, for praying for me. I write this thesis to say a big thank you for being my parents and to say I love you. Finally, the one above all of us, the omnipresent God, for answering my prayers and for giving me the strength to march on despite my wanting to give up and throw in the towel, thank you so much Dear God.

Table of Contents

Abstract.....	iii
Acknowledgments.....	v
Table of Contents.....	vii
List of Figures.....	ix
Introduction.....	1
I. Dichloroacetate (DCA) and Trichloroacetate (TCA).....	1
II. DCA and TCA and toxicity/carcinogenicity in mice.....	2
III. Phagocytic activation and oxidative stress.....	3
IV. DCA- and TCA-induced phagocytic activation.....	6
V. Vitamin E and oxidative stress.....	7
Objective.....	10
Materials and Methods.....	11
I. Chemicals.....	11
II. Animals and Treatments.....	11
III. Collection of the Peritoneal Lavage Cells.....	12
IV. Determination of TNF-Alpha (TNF- α).....	13
V. Determination of Superoxide Anion (SA).....	13

VI.	Determination of myeloperoxidase (MPO) activity.....	14
VII.	Determination of superoxide dismutase (SOD) activity.....	14
VIII.	Determination of catalase (CAT) activity	15
IX.	Determination of glutathione peroxidase (GSH-Px) activity.....	15
X.	Determination of Protein.....	16
XI.	Statistical Methods.....	16
	Results.....	17
	Discussion.....	19
	Future Studies.	24
	References.....	33

List of Figures

Figure 1. Activation of the neutrophil NADPH oxidase.....	4
Figure 2. Standard Curve for TNF- α	25
Figure 3. BSA Standard Curve	26
Figure 4. Superoxide anion production, in the PLCs of E-deficient and E-normal mice after 13 weeks exposure to DCA and TCA	27
Figure 5. Superoxide dismutase activity determined in the PLCs of E-deficient and E-normal mice after 13 weeks exposure to DCA and TCA	28
Figure 6. Glutathione peroxidase activity determined in the PLCs of E-deficient and E-normal mice after 13 week of exposure to DCA and TCA.....	29
Figure 7. Catalase activity determined in the PLCs of E-deficient and E-normal mice after 13 week exposure to DCA and TCA	30
Figure 8. Myeloperoxidase activity determined in the PLCs of E-deficient and E-normal mice after 13 weeks exposure to DCA and TCA.....	31
Figure 9. TNF- α levels determined in the PLCs of E-deficient and E-normal mice after 13 weeks of exposure to DCA and TCA.....	32

Introduction

I. Dichloroacetate (DCA) and Trichloroacetate (TCA)

Water chlorination is one of the most commonly used methods to control waterborne infectious diseases. However, chlorine was found to react with many natural organic materials in surface waters, producing an ample array of by-products, of which the haloacetates are regarded as the major components (Kato-Weinstein et al. 2001). Dichloroacetate (DCA) and trichloroacetate (TCA) are considered the most common haloacetates present in the finished chlorinated drinking water, and municipal water supplies are found to contain the compounds at concentration ranging from 30 to 160 $\mu\text{g/L}$ (Uden and Miller 1983).

Trichloroethylene (TCE) is one of the most common industrial solvents that has several applications (IARC, 1995; Kaneko et al., 1997; Maull et al., 1997), and the wide spread use of this solvent resulted in the contamination of ground water, surface water, and hazardous waste disposal sites (Coleman et al., 1984; Conglio et al, 1980; Westerick et al., 1984). TCE is believed to be metabolized into DCA and TCA in animals and humans (Bruckner et al., 1989; IARC, 1995), and therefore, humans are exposed to DCA and TCA as a result of drinking chlorinated water, as well as from possible *in vivo* metabolism of TCE.

II. DCA and TCA and toxicity/carcinogenicity in mice

Experiments on the carcinogenic potential of TCA and DCA in mice, found the compounds to affect primarily the liver. Administration of DCA and TCA in the drinking water to both male and female B6F1C3 mice, and male F344 rats was found to increase the incidence of liver cancer (Herren-Freund et al., 1987; DeAngelo et al., 1989, 1991, 1996; Bull et al., 1990). Although both compounds were found to induce hepatic tumors in the B6F1C3 male mice, the mechanisms of DCA and TCA hepatotoxic and hepatocarcinogenic effects are believed to be different (Herren-Freund et al., 1987; Bull et al., 1990; Tao et al., 1996). DCA and TCA have also been shown to induce peroxisome proliferation in the hepatic tissues (DeAngelo et al., 1986, IARC, 1995; Komulainen, 2004). While TCA is known to be a peroxisome proliferator in the liver of mice and rats (IARC, 1995), and peroxisome proliferation has been proposed as the major mechanism of TCA inducing hepatic cancer in mice (Komulainen, 2004), peroxisome proliferation by DCA occurs at doses significantly higher than those required to induce hepatic tumors (DeAngelo et al., 1989). In addition, TCA leads to increased rate of DNA replication which is associated with depleted S-adenosyl-methionine in the liver and is believed to lead to hypomethylation of DNA and the promoter regions of oncogenes such as c-jun and c-myc (Tao et al., 2000; Ge et al., 2001). This is also believed to amplify oncogene expression and *ras* genes mutation leads to TCA-induced liver tumors (Ferreira-Gonzales et al., 1995). DCA is also known to be a hepatocarcinogen in male and female mice (Komulainen, 2004), but it does so through increasing the rate of cell replication in liver foci and tumors (Stauber and Bull, 1997). Further studies on DCA carcinogenicity showed that by termination of DCA exposure, the growth of liver tumors desisted

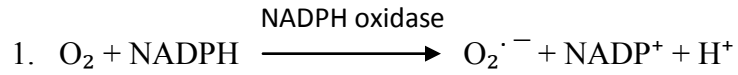
(Pereira and Phelps, 1996; Miller et al., 2000), signifying a promotion type effect.

However, other studies have found that DCA and TCA to act as complete carcinogens in B6C3F1 mouse liver (Herren-Freund et al.1987).

III. Phagocytic activation and oxidative stress

Even though oxygen is necessary for the humans' life, it is a risky molecule when converted to reactive species (ROS) inside the body and can damage various cellular components, such as proteins, lipids, and DNA (Nathan and Shiloh 2000). This condition is known as oxidative stress and is the result of imbalance between production of ROS and the body's ability to readily detoxify those reactive intermediates and repairing the resulting damage (Chow 1991). Although this process is destructive to the cells, the immune system uses it as a central part of its mechanism of killing invading pathogens. Phagocytes, including monocytes, macrophages, neutrophils, dendritic cells, and mast cells, are the first line of defense against invading pathogens by killing and disposing of them with remarkable effectiveness (Babior 2000). The use of these highly reactive compounds by phagocytic cells, although is essential for fighting infections and for subsequent immunity, they can cause damage to surrounding host tissues (Nathan and Shiloh 2000). Activation of phagocytic cells contributes to a significant increase in energy and oxygen consumption, a process known as the "respiratory burst" (Forman and Torres 2002), and is catalyzed by the action of three enzymes, including NADPH oxidase, superoxide dismutase (SOD), and myeloperoxidase (MPO). NADPH oxidase is a membrane- bound enzyme that consists of a number of subunits and can transfer one

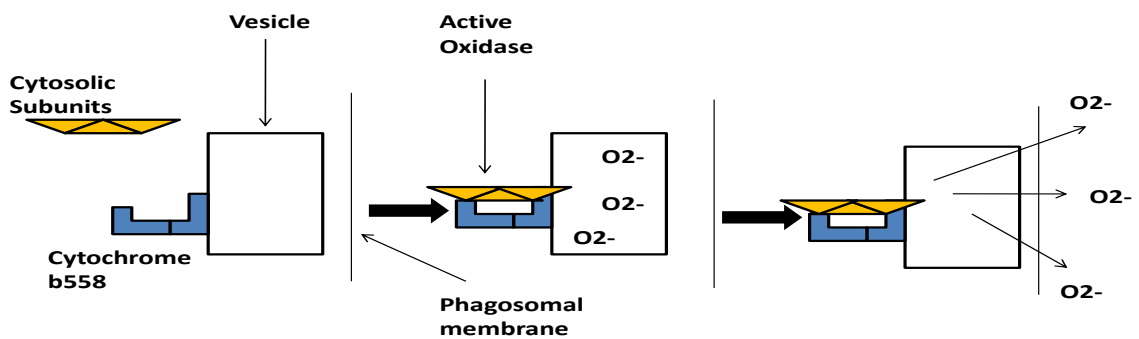
electron to molecular oxygen, yielding the superoxide radical ($O_2^{\cdot -}$) or superoxide anion (SA) (Fig.1).



In the resting cell, the enzyme's subunits are disturbed, but when the phagocyte is activated, the cytosolic subunits migrate to the vesicle's membrane, and bind to the membrane-associated cytochrome b558 subunit. The latter assembles the active NADPH oxidase, after which the intracellular vesicle fuses with the phagosomal membrane enveloping the cell lining the phagocytic vesicle resulting in SA delivery (Babior 2000).

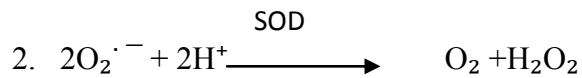
This superoxide radical, is produced in large quantities in the phagocytes for use in oxygen-dependent killing mechanisms of invading pathogens (Babior 2000).

Figure 1

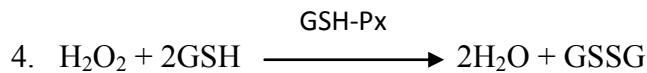
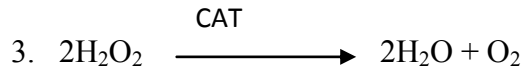


The SA is only moderately reactive and can rapidly react with itself, undergoing dismutation, generating oxygen and hydrogen peroxide (H₂O₂) (Babior 2000). The enzyme responsible for

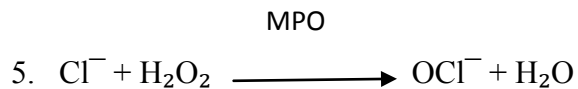
dismutation is SOD:



H₂O₂ can be converted by two antioxidant enzymes, namely glutathione peroxidase and catalase, to water, as follows:



Hydrogen peroxide can damage microbes, but can be converted by MPO found in the granules of the neutrophils to the more damaging hypochlorite (OCl⁻). Hypochlorite is a powerful antimicrobial agent that readily kills almost any microorganism (Klebanoff 2005):



TNF-α is one of the main pro-inflammatory cytokines and is also involved in many other biological processes such as antitumor effects and immune response to infection. The cytokine is also found to be produced by activated macrophages (Carswell et al., 1975; Belloni et al., 2000).

During the process of SA dismutation by SOD, hydrogen peroxide production takes place. Hydrogen peroxide is clearly toxic and must be rapidly removed. In mammalian

cells, the two antioxidant enzymes responsible for this are glutathione peroxidase (GSH-Px) and catalase (CAT) (Davis, 1995). Catalase activity is essential in dealing with large overproduction of hydrogen peroxide by converting it to water. Glutathione peroxidase is located in the cytosol, mitochondria, and nucleus. It catalyzes the reduction of hydrogen peroxide to water with glutathione acting as a co-substrate (Martinez-Cayuela, 1995). CAT is not commonly found in the cytoplasm of most mammalian cells, and since hydrogen peroxide diffusion from the cytoplasm into the peroxisomes is possibly unlikely, therefore, GSH-Px seems probable to largely deal with cytoplasmic hydrogen peroxidase whereas CAT is likely to deal with peroxisomal hydrogen peroxide (Davis, 1995).

IV. DCA- and TCA-induced phagocytic activation

In vitro studies in J774.1 macrophage cell cultures have demonstrated production of concentration- and time-dependent increases in SA in response to DCA and TCA, and that SA production was correlated with cellular death (Hassoun and Rey 2003, Hassoun and Kini 2004, Hassoun and Mehta 2008). The role of TNF- α in DCA- and TCA-induced production of SA and cellular death has been also assessed *in vitro*, in the same cell cultures, where TNF- α was found to provide a significant protective effect (Hassoun and Kini 2004, Hassoun and Mehta 2008). *In vitro* studies on the toxicity of DCA and TCA in the J744A.1 cells have also demonstrated the protective role of SOD against DCA and TCA induced production of SA and cellular death (Hassoun and Rey 2003).

Exposure of mice to acute single doses of DCA and TCA was found to be associated with phagocytic activation, assessed by SA production by the peritoneal

lavage cells (PLCs) of mice (Hassoun and Dey 2008). The studies have also demonstrated an association between phagocytic activation and the production of oxidative stress in the hepatic tissues of those same mice (Hassoun and Dey, 2008). Subacute and subchronic exposure of mice to DCA and TCA was found to induce phagocytic activation that was assessed by SA and TNF- α production, as well as by MPO activity in the peritoneal lavage cells (Hassoun et al. 2009). The studies have shown that DCA and TCA induce significant SA production in response to subacute and subchronic treatment with 77–410 mg/kg/day and 7.7–154 mg/kg/day, respectively. They have also demonstrated parallel increases in MPO activity and SA and TNF- α production, in response to DCA and TCA, upon both subacute and subchronic treatments. However, at doses that were proved to be hepatocarcinogenic, the biomarkers of phagocytic activation declined significantly, suggesting a protective rather than damaging role of this mechanism in the long term induction of hepatotoxicity/hepatocarcinogenicity by DCA and TCA.

V. Vitamin E and oxidative stress

Vitamin E is a fat-soluble vitamin that is synthesized by plants and consists of eight different vitamers. The vitamers are divided into two classes where each class has four, and are made up of a 6-chromanol ring and an isoprenoid side chain. While vitamers in the first class have saturated side chains and are labeled as tocopherols, the vitamers in the second class have unsaturated side chains and are classified as tocotrienols (trienols) (Groff et al 1995). Four different vitamers are currently identified and they are alpha, beta, gamma, and delta tocopherol. Most of the current research focuses on the most

biologically active form, which is the alpha tocopherol form of vitamin E (Tiidus et al. 1995, and Traber 1999). Vitamin E is the major antioxidant in membranes (Packer 1997), and is found to protect against oxidation that are linked to various diseases and conditions such as cancer, aging, arthritis, and many other diseases (Packer 1991). Vitamin E also acts as a cell membrane stabilizer (Meydani et al 1996, Tiidus et al. 1995) by allowing a tighter packing of the membrane and enhancing the degree of order of lipid packaging (Tiidus et al. 1995). A large number of chemicals and drugs have been shown to be capable of causing direct oxidative stress by generating free radical intermediates that seem to contribute to their toxic effects (Chow 1991). While depletion of dietary vitamin E was found to enhance the toxic effect of a number of chemicals and drugs (Walker et al. 1974, Ando and Tappel 1985, Martinez-Calva et al. 1984, Hong and Chow 2002), vitamin supplementation of diet was found to reduce the toxic effects in many cases (Walker et al. 1974, Ando and Tappel 1985, Martinez-Calva et al. 1984, Zidenberg-Cherr and Keen 1986, Cadet et al. 1989). Kawase et al. (1989) have shown that the levels of lipid peroxidation products and α -tocopheryl quinone were significantly higher in the liver of rats receiving low dietary vitamin E and chronically fed with ethanol, as compared with the controls receiving ethanol with normal diet. Adriamycin is a chemotherapeutic drug that is known to be effective against human cancers such as leukemias, lymphomas, and other solid tumors, and repeated exposure to the drug can lead to cardiomyopathy and heart failure, that is associated with production of free radicals (Lefrak et al., 1973, Singal et al., 1987). Singal and Tong (1988) reported increased mortality and suggestive symptoms of congestive heart failure, associated with increased lipid peroxidation in animals that were treated with adriamycin and were on

vitamin E-deficient diet. Copper is a potent hepatotoxic metal that was shown to be associated with production of reactive oxygen species and lipid peroxidation (Hochstein et al., 1980). While exposure to copper enhanced toxicity in vitamin E-deficient rat hepatocytes (Sokol et al., 1989), pretreatment with vitamin E was found to reduce lipid peroxidation in livers and decreased mortality in rats that are intraperitoneally injected with copper sulfate (Dougherty and Hoekstra, 1982). Long term treatment with prednisolone was found to lead to accumulation of the drug in the lens of the eye causing oxidation of the lens fiber and cataract formation, an effect that was shown to be enhanced under vitamin E deficiency conditions (Kojima et al., 2002). d-MDMA is a substituted amphetamine that was found to cause dopaminergic neurotoxicity in the mouse brain, but elevated levels of vitamin E reduced the drug's neurotoxicity (Logan et al., 1988). Also, Johnson et al. (2002) showed that d-MDMA induced neurotoxicity by producing free radical species, and feeding vitamin E deficient diet to mice increased the extent of neuronal damage.

Objectives

Depending on several conditions of exposure to different toxic xenobiotics, phagocytic activation may either contribute to the toxic effects of those xenobiotics, or to the protection against those effects. It has been previously shown that DCA and TCA induce phagocytic activation *in vivo*, in mice after subacute and subchronic treatments, and that this mechanism was suggested to contribute to the protection against, rather than to the hepatocarcinogenic effects of the compounds (Hassoun and Spildener, 2009).

Vitamin E on the other hand is an important antioxidant that protects against the induction of oxidative stress and toxic effects associated with phagocytic activation by several xenobiotics. Since phagocytic activation was suggested as a protective mechanism against the long term hepatotoxic/hepatocarcinogenic effects of DCA and TCA, marginal E deficiency was expected to suppress DCA- and TCA-induced phagocytic activation, increasing the hepatotoxic/hepatocarcinogenic effects of the compounds.

Materials and Methods

I. Chemicals

Sodium dichloroacetate (DCA) and sodium trichloroacetate (TCA), as well as all of the other chemicals used, were purchased from Sigma Chemical Company (St. Louis, MO) and were of the highest grades available.

II. Animals and Treatments

B6C3F1 male mice were purchased from Harlan (Indianapolis, IN) and used for this study, based on previous studies that found the animals to be sensitive to the acute, as well as the long-term effects of DCA and TCA (Herren-Freund et al., 1987, 1990; DeAngelo et al., 1991; Bull et al., 1990; Hassoun and Dey, 2008). The protocol for the use of animals for this proposal had been approved by the UT IACUC (protocol #: 105413), which covers the NIH guidelines for the animals use and handling. Upon receiving their first doses of DCA and TCA, the animals were about 6 weeks of age and weighing approximately 20 g. Prior to experimentation, the mice were given a period of 3 days to acclimate, at caged temperature of 21°C with a 12-h light/dark cycle. The animals were divided into two groups and were given different diets that were purchased from Harlan (Indianapolis, IN). The first group was maintained on a standard laboratory chow diet and was named the E-normal group, whereas the second group was maintained on low vitamin E diet (NIH-31 Mod) and named the E-deficient group. The animals were allowed free access of food and water. DCA and TCA were dissolved in distilled water, with pH of both solutions being adjusted to 7.0 using sodium hydroxide solution. Three

groups of mice (seven animals/group) were treated post orally, by gavage, with daily doses of 77 mg/kg body weight of DCA, TCA, or distilled water (Control), at a rate of 5.0 ml/kg body weight for 13 weeks. The dose of the compounds used for this study was based on previous long term studies demonstrating it to be the threshold dose for hepatotoxicity/ hepatocarcinogenicity in the B6C3F1 male mice (D'Angelo et al., 1991), as well as being able to induce significant oxidative stress in the hepatic tissues and phagocytic activation in the same animals, after 13 weeks of treatment (Hassoun et al., 2009; Hassoun and Cearfoss, 2010). At the end of the treatments, the mice were euthanized by carbon dioxide anesthesia, followed by cervical dislocation.

III. Collection of the Peritoneal Lavage Cells

A buffer containing 140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, and 2 mM CaCl₂ with pH adjusted to 7.3 was used for the collection of PLCs, where 3ml of which was injected into the peritoneal cavities of the animals, immediately after sacrifice. The peritoneal cavities were massaged in order to release the cells, and the fluids (PLC suspensions) were then withdrawn by a syringe. The PLC suspensions were centrifuged at 1700×g for 10 min, and the cell pellets were then re-suspended in 2 ml of Dolbeccos' Modified Eagle Medium (DMEM) containing methionine and supplemented with glutamine, penicillin-streptomycin, HEPES buffer, MEM-non essential amino acids, sodium pyruvate solution, and fetal bovine serum. Cellular suspensions were used for the determination of TNF- α and SA production, as well for the activities of MPO, SOD, CAT, and GSH-Px, as described below.

IV. Determination of TNF-Alpha (TNF- α)

Aliquots of 200 μ l of cellular suspensions were plated on a 96-well plate and were used to determine TNF- α . Cellular suspensions were plated on a 96-well plate were incubated for 24 h at 37°C in a humidified environment containing 5% CO₂. Media were then collected and used for the determination of TNF- α production, by using the ELISA Quantikine mouse TNF- α immunoassay (R & D Systems, Minneapolis, MN). The assay was carried out according to the procedure specified in the kit and plates were read in a microplate reader set to 450 nm. TNF- α concentrations were determined according to a standard curve prepared from a series of dilutions of recombinant murine TNF- α standard that was provided with the kit (Figure 2).

V. Determination of Superoxide Anion (SA)

The cytochrome *c* reduction assay of Babior et al (1973) was used to determine the SA production in the PLCs, with modifications. Basically, a 2 ml reaction mixture contained 50 μ l of PLCs suspension and 0.05 mM cytochrome *c* oxidase in phosphate buffered saline, pH being adjusted to 7.2. Tubes containing the reaction mixtures were incubated for 15 min at 37°C. Reactions were then stopped immediately by placing the tubes in crushed ice, and absorbances were determined in a Spectronic 20 spectrophotometer, at a wavelength of 550 nm. Absorbance values were converted to nanomoles of cytochrome *c* reduced/min, using the extinction coefficient $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

VI. Determination of myeloperoxidase (MPO) activity

The method of Bradley et al. (1982) was conducted to measure the activity of MPO. Aliquots of 0.5 ml cellular suspensions were freeze-thawed three times. The cell suspensions were centrifuged at 4000×g for 15 min, and the supernatant fractions were assayed. The assay mixture contained 0.1 ml of supernatant mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in the absorbance was measured at 460 nm for 5 min, and absorbance values were converted to units of MPO activity using a molar extinction coefficient of 11,300 for the oxidized *o*-dianisidine, where one unit is defined as that degrading 1 μmol of hydrogen peroxide per minute at 25°C.

VII. Determination of superoxide dismutase (SOD) activity

Aliquots of 0.5 ml cellular suspensions were centrifuged at 3000×g for 10 min, and cell pellets were re-suspended in 2 ml sucrose buffer containing 0.32 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl. Cellular suspensions were freeze-thawed three times and were then centrifuged at 4000×g for 15 min, and the supernatant fractions were assayed for SOD activity according to the method of Marklund and Marklund (1974). Briefly, 200 μl of the supernatant fraction was mixed with 750 μL Tris-cacodylic buffer, containing 50 mM Tris-HCl, 50 mM cacodylic acid, and 1 mM EDTA, pH 8.2, and 250 μl of 2 mM pyrogallol. After mixing, absorbances were immediately recorded at 420 nm over a period of 3 min, using a Spectronic 20 spectrophotometer. The calculated changes in the rate of absorbances were converted into units of SOD activity. One unit is defined

as the quantity of SOD that is needed to produce 50% inhibition of pyrogallol autooxidation .

VIII. Determination of catalase (CAT) activity

The method of Cohen et al (1970) was employed to measure the activity of CAT, with modifications. The method was based enzyme-catalyzed decomposition of H₂O₂, using potassium permanganate (KMnO₄). The assay consists of three components, the blank, standard, and sample. The blank reaction tube contained 100 µl of sucrose buffer, the standard tube contained 100 µl of deionized water, and the sample tube contained 100 µl of supernatant. One milliliter of 6 mM H₂O₂ was added to the sample and the blank tubes, and 1 ml of deionized water was added to the standard tube. The tubes were vortexed and placed on ice for 3 min, and reactions were then terminated by the addition of 200 µl of 6 N H₂SO₄. KMnO₄ at a concentration of 2 mM was added to each tube (1.4 ml/tube), and absorbances were immediately recorded at 480 nm, using a Biotek® Spectrophotometer. Absorbance values were converted into units of CAT/mg protein. One unit is equivalent to $k/0.00693$, where the rate constant $(k) = \log (S_0/S_2) \times (2.3/t)$. S₀ and S₂ were determined by subtracting the blank absorbance and sample absorbance, respectively, from the standard absorbance, t is equal to the time of incubation (3 min) and 2.3 is the logarithm conversion factor.

IX. Determination of glutathione peroxidase (GSH-Px) activity

GSH-Px activity was measured according to the method of Lawrence and Burk (1976).

This method is conducted as follows: a 100 µl aliquot of supernatant was mixed with 700

μl of reaction mixture containing 1 mM EDTA, 1 mM NaN₃, 0.2 mM NADPH, 1 mM GSH in a phosphate buffer saline (pH 7.2), and 10 units of glutathione reductase. The tube mixtures were vortexed and incubated for 5 min at room temperature. The reaction was immediately initiated after incubation by the addition of 100 μl of 0.2 mM.

Absorbance was recorded over a period of 3 min at a 30 sec interval, using a Spectronic 20 spectrophotometer (Spectronic Instruments, Rochester, NY). Changes in the rate of absorbance were converted into nmoles of NADPH oxidized/min/mg protein, using an extinction coefficient of $6.22 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ (Lawrence and Burk, 1976)

X. Determination of Protein

The amounts of protein in PLC suspensions were determined according to the method of Lowry (1951) , using bovine serum albumin as a standard (figure 3), and data for SA, SOD, MPO, and TNF- α , were expressed as nmol/min, units, units, and pg, respectively, /mg protein.

XI. Statistical Methods

Data were analyzed using Microsoft Excel data analysis tool package. Data are expressed as means of seven samples (animals)/treatment in each diet group \pm SD. A single-factor analysis of variance (ANOVA) was used to determine the statistical differences between two groups at a time, and a significance level of $p < 0.05$, was employed for all of the groups.

Results

Figure 4 demonstrates SA production in the PLCs in response to DCA and TCA treated mice that are kept on E-deficient diet and E-normal diet. No significant difference was observed between the controls of the two groups. Significant increases were observed in response to DCA and TCA in both diet groups, when compared with the corresponding controls. However, SA levels in the E-deficient group treated with DCA and TCA were significantly higher when compared with those of the corresponding treatments in the E-normal group.

Figure 5 illustrates the activity of SOD in the PLCs, after treatment with DCA and TCA. DCA and TCA treatment of both, the E-deficient and the E-normal diet groups resulted in significant increases in SOD activity, as compared with the corresponding controls in both diet groups. However, the observed increases in SOD activity in response to both compounds in the E-deficient groups were significantly greater, when compared with the corresponding treatments in the E-normal group.

The effects of treatment of mice with DCA and TCA on the PLCs' GSH-Px activity are demonstrated in Figure 6. In the E-normal groups, neither compounds resulted in significant changes in GSH-Px activity when compared with the corresponding control. However, both DCA and TCA treatment resulted in significant increases in GSH-Px activity in the E-deficient group, as compared with the corresponding control. The figure also shows that the DCA- and TCA-induced increase in GSH-Px activity in the E-deficient groups were significantly greater than those observed in the E-normal groups.

The effects of DCA and TCA on CAT activity in the PLCs are represented in Figure 7. No significant difference in CAT activity was observed when comparing the controls of the two diet groups. Also, neither compound resulted in significant change in CAT activity in the E-normal group when compared with the corresponding control. However, both DCA and TCA treatments resulted in significant increases in CAT activity in the E-deficient groups, as compared with the corresponding treatments in the E-normal groups.

Figure 8 demonstrates the effects of DCA and TCA on the MPO activity in the PLCs. No significant change in MPO activity was observed when comparing the control in the E-normal group with the control in the E-deficient group. However, both compounds resulted in significant increases in MPO activity in the E-normal group and the E-deficient group when compared with the corresponding controls, with significantly greater increases observed in the E-deficient groups as compared with the corresponding treatments in the E-normal groups.

The effects of DCA and TCA treatment on TNF- α production in the PLCs are illustrated in Figure 9. TNF- α levels were found to be significantly high in response to both compounds in the two diet groups when compared with the corresponding controls. Also, the observed increases in response to DCA and TCA in the E-deficient group were significantly higher than those observed with the corresponding treatments in the E-normal groups.

Discussion

Previous studies have found that subchronic exposure of B6C3F1 mice to 77 mg DCA or TCA /kg/day had the most significant effects on various biomarkers of phagocytic activation , including SA, MPO activity and TNF- α , as compared with the other tested doses (Hassoun et al, 2009). Therefore this dose was chosen for the study, and the observed increases in SA, MPO activity and TNF- α in the E-normal group are consistent with those of the previous ones.

Groups of mice were placed on (NIH-31 Mod) diet, which is similar to the normal standard diet but was not supplemented with vitamin E, i.e., vitamin E was only provided by the natural ingredient of the diet and its level was in the neighborhood of 20-30 IU/kg diet, compared to the estimated 90 IU/kg level in the standard diet. At that level of vitamin E supplementation to the diet, optimal immunopotentiality, growth, and reproduction are achieved in mice (Yasunaga et al, 1982, personal communication with the nutrition expert at Harlan). Although severe E deficiency in animals is known to induce myopathy (Nier et al, 2006; Thomas et al., 1993; Gabriel et al., 1984; Jackson et al., 1983), the E level in the diet not supplemented with the vitamin placed the animals on marginal E deficiency, which is only known to contribute to effects on the reproductive ability of the animals (personal communication with the nutritional expert in Harlan, Bensoussan et al, 1998, Raychaudhuri and Desai, 1971). This was confirmed by observations on the behaviours of animals and their weight gain that revealed no significant adverse health effects in the E-deficient diet groups, as compared with the E-normal groups during the 13 weeks of treatment period.

Increase in MPO activity and TNF- α are biomarker of phagocytic activation, and our results clearly indicate significant increases in those biomarkers in both the E-normal and the E-deficient diet groups. Wang and Lin (2008) have described this cytokine as a “double-dealer”, where it can act as an endogenous tumor promoter by initiating the nuclear factor- κ B, or a cancer killer by initiating c-Jun N-terminal kinase that is considered as a cell death signal. Previous studies suggested the contribution of phagocytic activation in general, and MPO and TNF- α , in particular to the long term hepatotoxicity and hepatocarcinogenicity of DCA and TCA and demonstrated that this mechanism is an early adaptive and protective mechanism (Hassoun et al., 2009). Thus, our current results suggest that marginal vitamin E deficiency promotes this mechanism to further protect against the long term effects of DCA and TCA.

SA production is another biomarker of phagocytic activation, and our results are showing significantly greater production of this ROS in PLCs from DCA- and TCA-treated mice maintained on E-deficient diet, as compared with the E-normal diet groups. These results further confirm the role of marginal vitamin E deficiency in enhancing the mechanism of phagocytic activation in response to the compounds.

SOD is responsible for SA dismutation to H₂O₂ and the significant increases in SOD in response to both compounds suggest significant production of H₂O₂ in either diet group treated with DCA or TCA. CAT and GSH-Px act in concert with SOD and catalyze conversion of H₂O₂ to water (Davies, 1995). Therefore, the no significant changes in CAT and GSH-Px activities in response to either compound in the E-normal groups suggest non significant roles of the enzymes in the protection against H₂O₂

overproduction, while the significant increases in those enzymes in the E-deficient groups suggest H₂O₂ detoxification in those groups. Since SA levels and SOD activities in the E-deficient groups are significantly greater than the corresponding increases in the two biomarkers in the E-normal group, it is expected to generate significantly greater H₂O₂ concentrations in the E-deficient groups than the E-normal ones. Therefore the increases in CAT and GSH-Px activities in the E-deficient groups may not be sufficient to keep up with H₂O₂ overproduction through SA dismutation in the E-deficient groups. H₂O₂ is a substrate for MPO (Josephy, 1996; Bradley et al., 1982; Lefkowitz et al., 1992; Kettle and Winterbourn, 1988), and the results showing significantly greater increases in MPO activity in the E-deficient groups treated with the compounds when compared with the corresponding E-normal groups may confirm our suggestion. Also, MPO reaction with H₂O₂ in the presence of physiological concentrations of Cl⁻ results in the formation of the powerful oxidant hypochlorous acid/hypochlorite (HOCl/OCl⁻) (Josephy, 1996; Bradley et al., 1982; Lefkowitz et al., 1992; Kettle and Winterbourn, 1988), and components of the peroxidase-H₂O₂-halide system, including MPO have been shown to contribute to the killing of certain mammalian tumor cells (Kettle and Winterbourn, 1989). Hence, these may all confirm our suggestion about the contribution of marginal vitamin E deficiency to the protective role of phagocytic activation against DCA and TCA long term effects.

Vitamin E is a major antioxidant that is shown to protect against oxidative stress and its associated cellular damage, including some effects on the phagocytes that may improve the immune system (de la Fuente et al, 1998). Therefore, and at the time this study was initiated, it was expected that marginal vitamin E deficiency may decrease DCA- and TCA-induced phagocytic activation. However, and contrary to what was expected, the

results are showing significant increases in the biomarkers of phagocytic activation in mice treated with the compounds when maintained on E-deficient diet, as compared with the treated groups maintained on E-normal diet. Further revision of literature as to the contribution of vitamin E to the process of phagocytic activation has revealed different findings. For example, studies by Eskew et al (1984) showed that dietary deficiency of vitamin E and selenium had no effects on alveolar macrophage function in rats, but studies by Moriguchi et al (1989) indicated the induction of higher phagocytic function of alveolar macrophages of rats in response to vitamin E deficiency. Similarly, studies by Fuente et al (2000) showed that low vitamin E diet in guinea pigs could increase the phagocytic activity of PLCs that was not associated with increase in the microbicidal activity, but studies by Baehner et al (1982) indicated that the ability of polymorphonuclear cells to ingest particles increases and bactericidal potency mildly decreases in humans receiving doses of vitamin E. Also, Sabat et al (2008) found that vitamin E deficiency can cause non mitochondrial-mediated increase in ROS formation in type II pneumocytes, pulmonary macrophages, and lymphocytes of rats, but short-term supplementation of vitamin E did not reverse these effects. Therefore it is not surprising that the observed changes in PLCs in response to marginal E deficiency of this study contradicted our expectations.

Chang et al (2007) could show that vitamin E deprivation in mice increases the exercise-induced elevation of the muscles' GSH-Px activity. Also, Tchanchou et al (2004) examined the separate and combined impacts of deprivation of folate and vitamin E coupled with dietary iron as prooxidant on mice, and found that the levels of glutathione was increased when mice were deprived of folate and vitamin E, suggesting deficiency in

one gene can result in compensatory up-regulation in a second relevant gene. Knowing that glutathione is a substrate for GSH-Px, the observed increases in GSH-Px activities in the E-deficient groups may be associated with increases in the levels of glutathione. However, further studies to determine the levels of glutathione in the PLCs of the treated mice are needed to confirm that.

In conclusion, the increases in antioxidant enzyme activities in the E-deficient groups treated with DCA or TCA may be viewed as compensatory up-regulation to provide protection, which is otherwise provided by vitamin E supplementation to the diet. It is also concluded here that supplementation of the animals' diet with vitamin E, while proved to be important to maintain optimal growth, fertility and reproduction, may compromise the antioxidant enzyme functions in PLCs of mice treated with DCA and TCA. However, the relative protection provided by compensatory up-regulation of antioxidant enzyme activities to that provided by vitamin E-supplementation against DCA- and TCA-induced hepatotoxic/hepatocarcinogenic effects need to be further investigated.

Future Studies

1. Marginal vitamin E deficiency is shown to enhance the induction of phagocytic activation by DCA and TCA. Since it has been previously suggested that DCA- and TCA-induced phagocytic activation contributes to the protection against DCA- and TCA-induced hepatocarcinogenicity/hepatotoxicity, E deficiency is expected to result in decreases in the incidences and rates of those effects in response to the compounds. Further studies to investigate production of those effects in the livers of E-deficient mice are required to assess that.

2. While we show here that antioxidant enzyme activities were increased in response to DCA and TCA when developed marginal E-deficiency and assume significant SA dismutation and H₂O₂ inactivation, the actual concentrations of H₂O₂ need to be determined in the PLCs of those groups, since SA level was also increased significantly in the E-deficient group. Determination of the levels of this ROS, in addition to liver toxicity/ carcinogenicity in the two diet groups exposed to DCA and TCA will help assess the contribution of the increase in antioxidant enzyme activity/ phagocytic activation in response to marginal E deficiency to the protection against DCA and TCA effects, as compared with the protection provided by vitamin E supplementation to the normal diet.

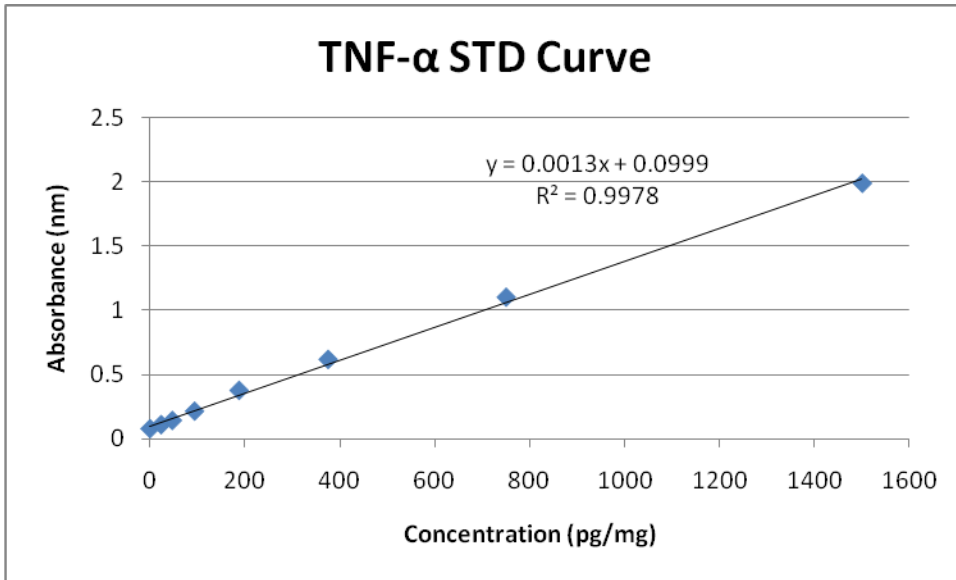


FIGURE 2. Standard Curve for TNF- α

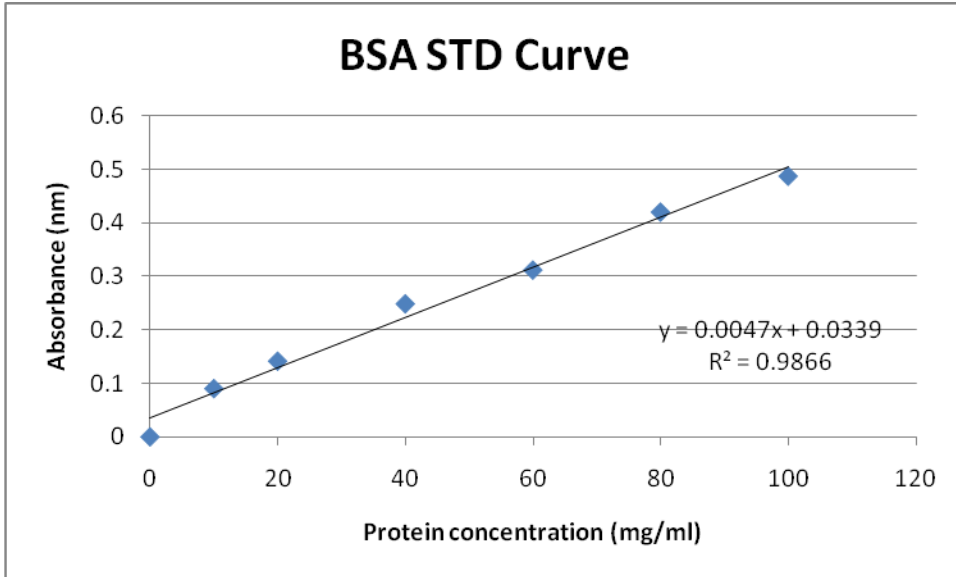


FIGURE 3. BSA Standard Curve.

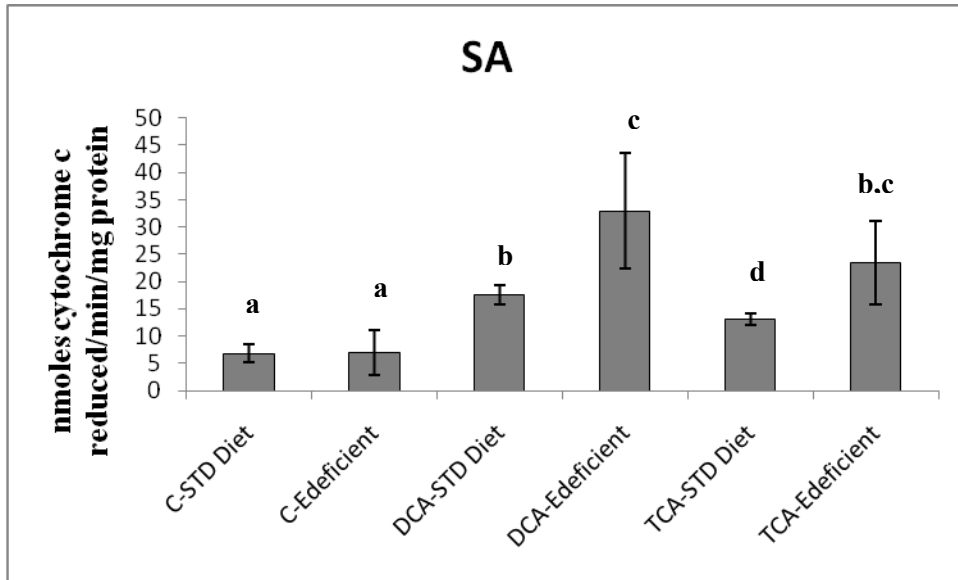


FIGURE 4. SA production, determined as cytochrome c reduced/min/mg protein in the PLCs of E-deficient and standard diet (E-normal) mice after 13 weeks exposure to DCA and TCA. Columns that do not share identical superscripts are significantly different ($p < 0.05$).

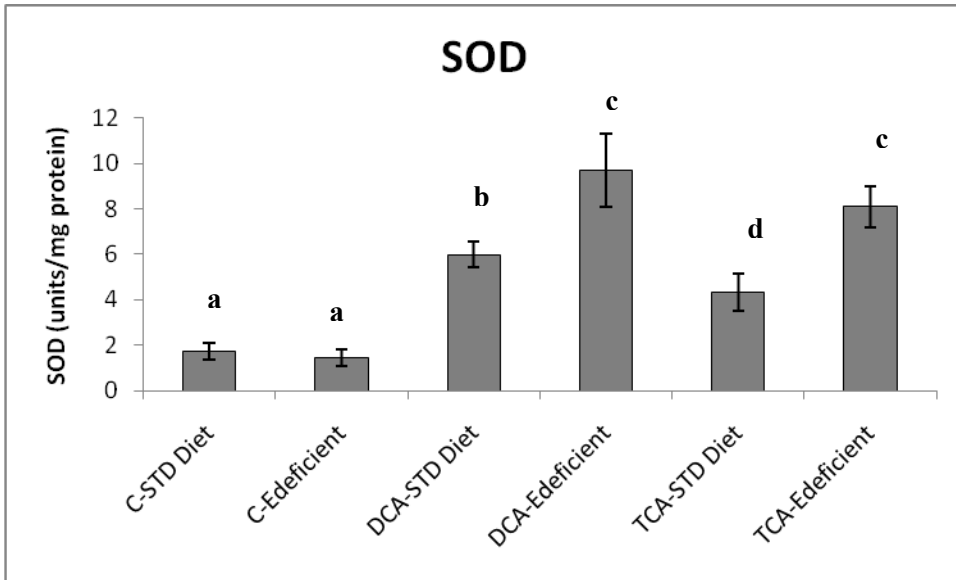


FIGURE 5. SOD activity determined in the PLCs of E-deficient and standard diet (E-normal) mice after 13 weeks exposure to DCA and TCA. Columns with non identical superscripts are significantly different ($p < 0.05$).

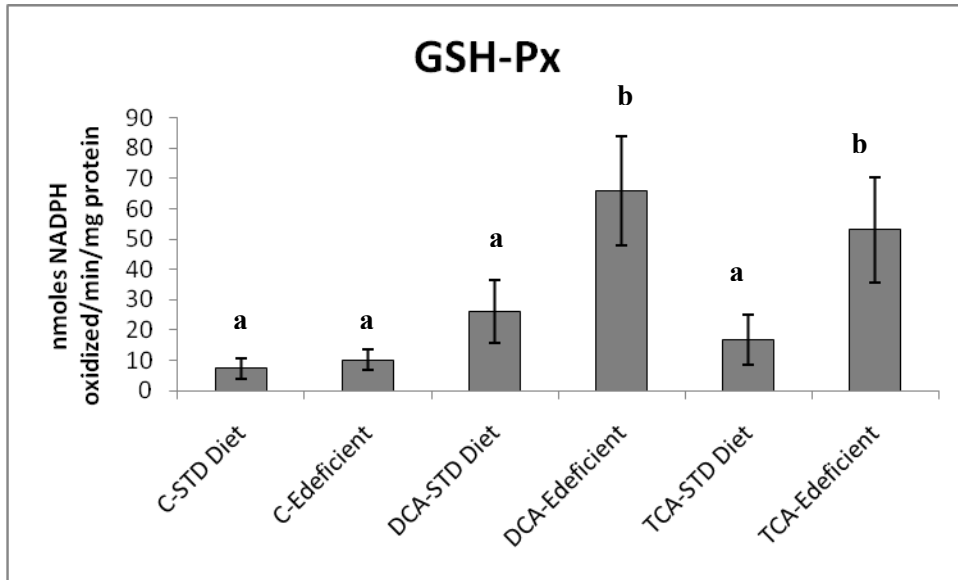


FIGURE 6. GSH-Px activity determined in the PLCs of E-deficient and standard diet (E-normal) mice after 13 week of exposure to DCA and TCA. Columns with non identical superscript are significantly different ($p < 0.05$).

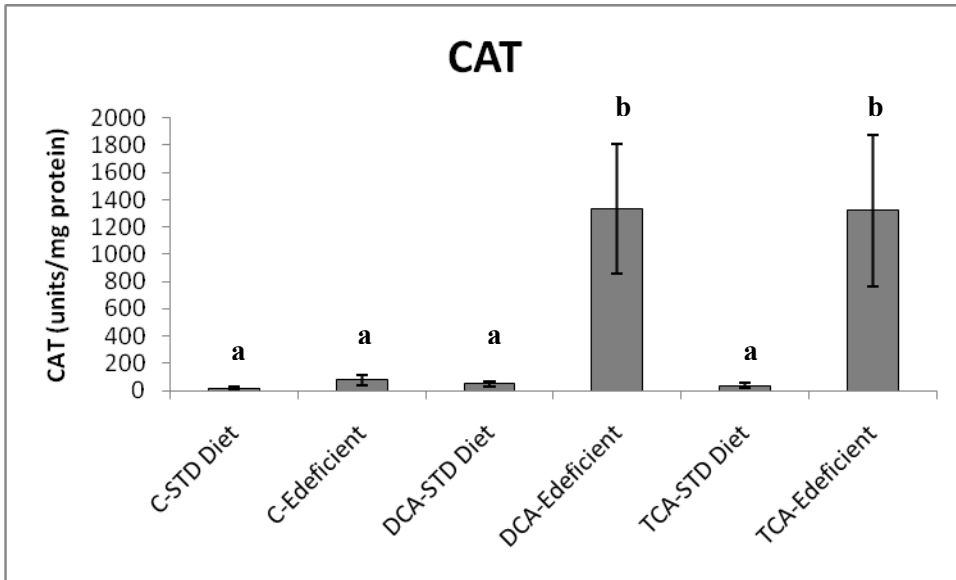


FIGURE 7. CAT activity determined in the PLCs of E-deficient and standard diet (E-normal) mice after 13 week exposure to DCA and TCA. Columns with non identical superscript are significantly different ($p < 0.05$).

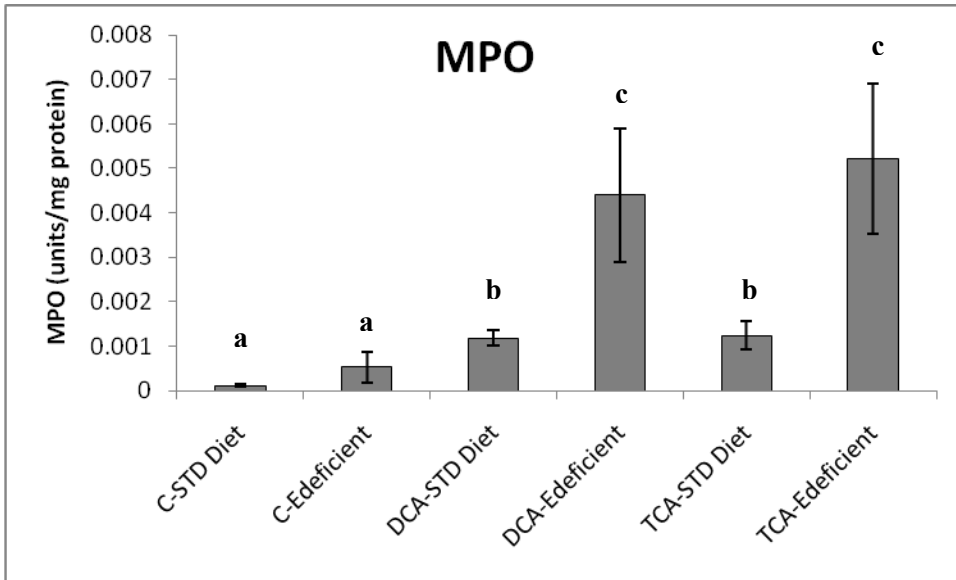


FIGURE 8. MPO activity determined in the PLCs of E-deficient and standard diet (E-normal) mice after 13 weeks exposure to DCA and TCA. Columns with non identical superscript are significantly different ($p < 0.05$).

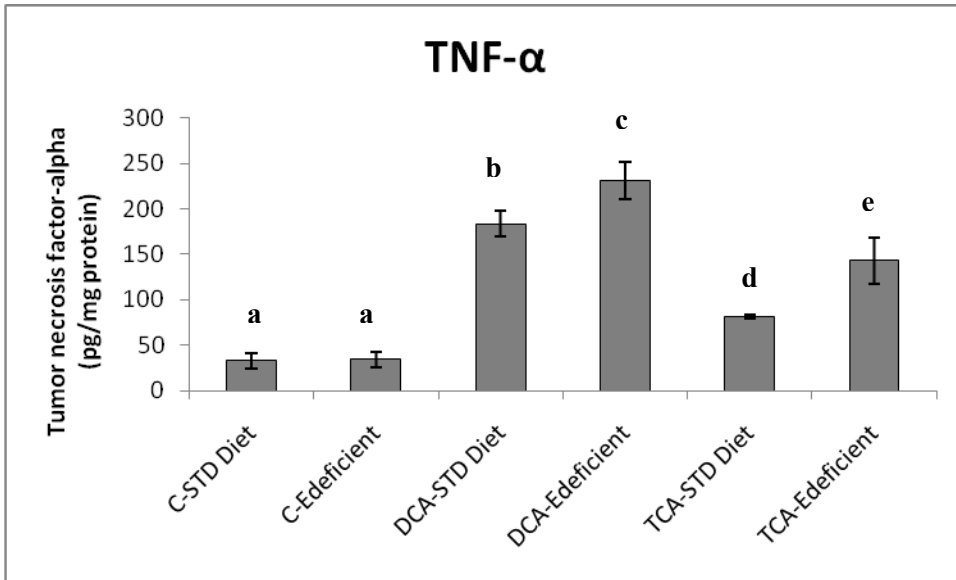


FIGURE 9. TNF- α levels determined in the PLCs of E-deficient and standard diet (E-normal) mice after 13 weeks of exposure to DCA and TCA. Columns with non identical superscript are significantly different ($p < 0.05$).

References

- Ando, M., and A. Tappel. "Effect of dietary vitamin E on methyl ethyl ketone peroxide damage to microsomal cytochrome P-450 peroxidase." Chemico-Biological Interactions 55 (1985): 317-26.
- Babior, Bernard M., Ruby S. Kipnes, and John T. Curnutte. "Biological Defense Mechanisms. THE PRODUCTION BY LEUKOCYTES OF SUPEROXIDE, A POTENTIAL BACTERICIDAL AGENT." Journal of Clinical Investigation 52 (1973): 741-44.
- Babior, BM. "Phagocytes and oxidative stress." Am J Med 109 (2000): 33-44.
- Baehner, Robert L., Laurence A. Boxer, Leah M. Ingraham, Charles Butterick, and Richard A. Haak. "The Influence Of Vitamin E On Human Polymorphonuclear Cell Metabolism And Function." Annals of the New York Academy of Sciences 393 (1982): 237-50.
- Belloni, A., D Aubert, JE Gomez Marin, R Le Naour, A Bonhomme, M Guenounou, and JM Pinon. "Involvement of tumor necrosis factor- α during infection of human monocytic cells by *Toxoplasma gondii*." Parasitol. Res 86 (2000): 406–412.
- Bensoussan, K., Morales, C. R. and Hermo, L. "Vitamin E deficiency causes incomplete spermatogenesis and affects the structural differentiation of epithelial cells of the epididymis in the rat." J. Androl. 19 (1998): 266-288.

- Bradley, PP, Priebat DA, Christensen RD, Rothstein G. "Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker." J Invest Dermatol 78 (1982): 206–209.
- Bruckner JV, Davis BD, Blancato JN. "Metabolism, toxicity, and carcinogenicity of trichloroethylene." Crit Rev Toxicol. 20 (1989): 31-50.
- Bull, R., I. Sanchez, M. Nelson, J. Larson, and A. Lansing. "Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate." Toxicology 63 (1990): 341-59.
- Cadet, J., M. Katz, V. Jackson-Lewis, and S. Fahn. "Vitamin E attenuates the toxic effects of intrastriatal injection of 6-hydroxydopamine (6-OHDA) in rats: Behavioral and biochemical evidence." Brain Research 476 (1989): 10-15.
- Carswell, E. A., LJ Old, S. Green, N. Fiore, and B. Williamson. "An Endotoxin-Induced Serum Factor that Causes Necrosis of Tumors." Proceedings of the National Academy of Sciences 72 (1975): 3666-670.
- Chow, CK, and CB Hong. "Dietary vitamin E and selenium and toxicity of nitrite and nitrate." Toxicology 180 (2002): 195-207.
- Chow, CK. "Vitamin E and oxidative stress." Free Radical Biology and Medicine 11 (1991): 215-32.
- Cohen, G., D. Dembiec, and J. Marcus. "Measurement of catalase activity in tissue extracts*1." Analytical Biochemistry 34 (1970): 30-38.

- Coleman, WE, Lingg RD, Melton RG, Kopfler FC. The occurrence of volatile organics in five drinking water supplies using gas chromatography/mass spectroscopy. Identification and analysis of organic pollutants of water. Ann Arbor, MI: Ann Arbor Science, 1976, pp. 305–327.
- Conglio, W. A., Miller, K., and Mackeener, D. (1980). The Occurrence of Volatile Organics in Drinking Water. Criteria and Standards Division, Science and Technology Branch, Environmental Protection Agency, Washington, DC.
- Davies, K. J. A. "Oxidative stress: the paradox of aerobic life." Biochem. Soc. Symp. 61 (1995): 1–31.
- DeAngelo, A.B., F.B. Daniel, B.M. Most, and G.R. Olson. "The carcinogenicity of dichloroacetic acid in the male fischer 344 rat." Toxicology 114 (1996): 207-21.
- DeAngelo, A.B., F.B. Daniel, J.A. Stober, and G.R. Olson. "The carcinogenicity of dichloroacetic acid in the male B6C3F1 mouse." Fundamental and Applied Toxicology 16 (1991): 337-47.
- DeAngelo, A.B., F.B. Daniel, L. McMillan, P. Wernsing, and R.E. Savage Jr. "Species and strain sensitivity to the induction of peroxisome proliferation by chloroacetic acids*1." Toxicology and Applied Pharmacology 101 (1989): 285-98.
- Delafuente, M., Ferrandez, M. D., Burgos, M. S., Soler, A., Prieto, A., Miquel, J. "Immune function in aged women is improved by ingestion of vitamins C and E." Can. J. Physiol. Pharmacol. 76 (1998): 373–380.

- Dougherty, JJ and Hoekstra, WG. "Effects of vitamin E and selenium on copper induced lipid peroxidation in vivo and on copper toxicity." Proc Sot Exp Biol Med 169 (1982): 201-208.
- Eskew, ML, RW Scholz, CC Reddy, DA Todhunter, A Zarkowr. Effects of vitamin E and selenium deficiencies on rat immune function." Immunology 54 (1985): 173-180.
- Ferreira-Gonzalez A, DeAngelo AB, Nasim S, Garrett CT. „Ras oncogene activation during hepatocarcinogenesis in B6C3F, male mice by dichloroacetic and trichloroacetic acids." Carcinogenesis 16 (1995): 495-500.
- Forman, H. J., and M. Torres. "Reactive Oxygen Species and Cell Signaling: Respiratory Burst in Macrophage Signaling." American Journal of Respiratory and Critical Care Medicine 166 (2002): 4S-8.
- Ge, Rongrong, Siming Yang, Paula M. Kramer, Lianhui Tao, and Michael A. Pereira. "The effect of dichloroacetic acid and trichloroacetic acid on DNA methylation and cell proliferation in B6C3F1 mice." Journal of Biochemical and Molecular Toxicology 15 (2001): 100-06.
- Groff, J.L., Gropper S.S., and Hunt S.M. The Fat Soluble Vitamins. In: Advanced Nutrition and Human Metabolism. Minneapolis: West Publishing Company, 1995, pp. 284-324.
- Hassoun EA, Cearfoss J. "Dichloroacetate- and Trichloroacetate-Induced Modulation of Superoxide Dismutase, Catalase, and Glutathione Peroxidase Activities and

Glutathione Level in the livers of Mice after Subacute and Subchronic exposure.”
Toxicol Environ Chem. 93 (2011): 332-344.

Hassoun EA, Mehta J. “Dichloroacetate-induced modulation of cellular antioxidant enzyme activities and glutathione level in the J774A.1 cells.” J Appl Toxicol. 8 (2008): 931-7.

Hassoun EA, Spildener J, Cearfoss J. “The induction of tumor necrosis factor-alpha, superoxide anion, myeloperoxidase, and superoxide dismutase in the peritoneal lavage cells of mice after prolonged exposure to dichloroacetate and trichloroacetate.” J Biochem Mol Toxicol. 24 (2010):1 36-44.

Hassoun, E., and S. Rey. "The induction of oxidative stress and cellular death by the drinking water disinfection by-products, dichloroacetate and trichloroacetate in J774.A1 cells." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 135 (2003): 119-28.

Hassoun, E., and V. Kini. "Effects of superoxide dismutase and polyclonal tumor necrosis factor-alpha antibodies on chloroacetate-induced cellular death and superoxide anion production by J774.A1 macrophages." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 138 (2004): 113-20.

Hassoun, Ezdihar A., and Soumyadeep Dey. "Dichloroacetate- and trichloroacetate-induced phagocytic activation and production of oxidative stress in the hepatic

tissues of mice after acute exposure." Journal of Biochemical and Molecular Toxicology 22 (2008): 27-34.

Herren-Freund, S.I., M.A. Pereir, M.D. Khoury, and G.R. Olson. "The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver." Toxicology and Applied Pharmacology 90 (1987): 183-89.

Hochstein, Paul, K. Sree Kumar, and Stephen J. Forman. "Lipid Peroxidation And The Cytotoxicity Of Copper." Annals of the New York Academy of Sciences 355 (1980): 240-48.

International Agency for Research on Cancer (IARC). Trichloroethylene. In IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 63. Lyon, France: 1995, pp.75–158.

Johnson, E., A. Shvedova, E. Kisin, J. Ocallaghan, C. Kommineni, and D. Miller. "D-MDMA during vitamin E deficiency: effects on dopaminergic neurotoxicity and hepatotoxicity." Brain Research 933 (2002): 150-63.

Joseph, PD. The respiratory burst. In Molecular toxicology. New York: Oxford University Press, 1996, pp. 90–96.

Kaneko, Takashi, Pei-Yu Wang, and Akio Sato. "Assessment of the Health Effects of Trichloroethylene." Industrial Health 35 (1997): 301-24.

- Kato-Weinstein, J., A. J. Stauber, G. A. Orner, B. D. Thrall, and R. J. Bull. "Differential effects of dihalogenated and trihalogenated acetates in the liver of B6C3F1 mice." Journal of Applied Toxicology 21 (2001): 81-89.
- Kawase, Tateo, Shinzo Kato, and Charles S. Lieber. "Lipid peroxidation and antioxidant defense systems in rat liver after chronic ethanol feeding." Hepatology 10 (1989): 815-21.
- Kettle, AJ, Winterbourn CC. "Influence of superoxide on myeloperoxidase kinetics measured with a hydrogen peroxide electrode." Biochem J 263 (1989): 823–828.
- Kettle, AJ, Winterbourn CC. "Superoxide modulates the activity of myeloperoxidase and optimizes the production of hypochlorous acid." Biochem J 252 (1988): 529–536.
- Klebanoff, S. J. "Myeloperoxidase: friend and foe." Journal of Leukocyte Biology 77 (2005): 598-625.
- Kojima M, Shui YB, Murano M, Nagata O, Hockwin K, Sasaki K, et al. "Low vitamin E level as a subliminal risk factor in a rat model of prednisolone-induced cataract." Investig Ophthalmol Visual Sci. 43 (2002): 1116–20.
- Komulainen, H. "Experimental cancer studies of chlorinated by-products." Toxicology 198 (2004): 239-48.
- Lawrence, R., and R. Burk. "Glutathione peroxidase activity in selenium-deficient rat liver*1." Biochemical and Biophysical Research Communications 71 (1976): 952-58.

- Lefkowitz DL, Mills K, Morgan D, Lefkowitz SS. "Macrophage activation and immunomodulation by myeloperoxidase." Proc Soc Exp Biol Med 199(1992): 204–210.
- Lefrak, Edward A., Jan Piřha, Sidney Rosenheim, and Jeffrey A. Gottlieb. "A clinicopathologic analysis of adriamycin cardiotoxicity." Cancer 32 (1973): 302-14.
- Logan, B.J., R. Lavery, W.D. Sanderson, Y.B. Yee. "Differences between rats and mice in MDMA (methylenedioxymethylam- phetamine) neurotoxicity." Eur. J. Pharmacol. 152 (1988): 227–234.
- Lowry, OH, Rosebrough NJ, Farr WL, Randall RJ. "Protein measurements with the folin phenol reagent." J Biol Chem 193 (1951): 265–275.
- Marklund S, Marklund G. "Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase." Eur JBiochem 47 (1974): 469–474.
- Martínez-Calva, I., A. Campos- Apáez, E. Rosales-Vega, and Marisabel Mourellet. "Vitamin E improves membrane lipid alterations induced by CCl4 intoxication." Journal of Applied Toxicology 4 (1984): 270-72.
- Martínez-Cayuela, M. "Oxygen free radicals and human disease." Biochimie 77 (1995): 147-61.

- Maull, E. A., Cogliano, V. J., Scott, C. S., Barton, H.A., Fisher, J. W., Greenberg, M., Rhomberg, L., and Sorgen, S. P. "Trichloroethylene health risk assessment: A new and improved process." Drug Chem. Toxicol. 20 (1997): 427– 42.
- Meydani, M., R.A. Fielding, N. Fotouhi. Vitamin E. In: Sports Nutrition Vitamins and Trace Minerals. New York: CRC Press, 1996, pp. 119-131.
- Miller JW, Uden PC. "Characterization of nonvolatile aqueous chlorination products of humic substances." Environ Sci Technol 17 (1983): 150–157.
- Moriguchi, S, Kobayashi N, Kishino Y. "High dietary intakes of vitamin E and cellular immune functions in rats." J Nutr 10 (1990): 96-102.
- Nathan, C. and Shiloh, M. U. "Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens." Proc. Natl. Acad. Sci. USA 97 (2000): 8841–8848.
- Nier, Bettina, Peter Weinberg, Gerald Rimbach, Elisabeth Stöcklin, and Luca Barella. "Differential gene expression in skeletal muscle of rats with Vitamin E deficiency." IUBMB Life (International Union of Biochemistry and Molecular Biology: Life) 58 (2006): 540-48.
- Packer, L. "Protective role of vitamin E in biological systems." Am. J. Clin. Nutr 53 (1991): 1050-1055.
- Packer, L. :Oxidants, antioxidant nutrients and the athlete." J. Sports Sci 15 (1997): 353-363.

- Raychaudhuri, C., and I. D. Desai. "Ceroid Pigment Formation and Irreversible Sterility in Vitamin E Deficiency." Science 173 (1971): 1028-029.
- Sabat, Robert, Florian Guthmann, and Bernd Rüstow. "Formation of Reactive Oxygen Species in Lung Alveolar Cells: Effect of Vitamin E Deficiency." Lung 186 (2008): 115-22.
- Singal, P., C. Deally, and L. Weinberg. "Subcellular effects of adriamycin in the heart: A concise review." Journal of Molecular and Cellular Cardiology 19 (1987): 817-28.
- Singal, Pawan K., and Jianguang Tong. "Vitamin E deficiency accentuates adriamycin-induced cardiomyopathy and cell surface changes." Molecular and Cellular Biochemistry 84 (1988): 163-71.
- Sokol, Ronald J., Michael W. Devereaux, Maret G. Traber, and Robert H. Shikes. "Copper Toxicity and Lipid Peroxidation in Isolated Rat Hepatocytes: Effect of Vitamin E." Pediatric Research 25 (1989): 55-62.
- Stauber, AJ, and RJ Bull. "Differences in Phenotype and Cell Replicative Behavior of Hepatic Tumors Induced by Dichloroacetate (DCA) and Trichloroacetate (TCA)." Toxicology and Applied Pharmacology 144 (1997): 235-46.
- Tao, L., K. Li, PM Kramer, and MA Periera. "Loss of heterozygosity on chromosome 6 in dichloroacetic acid and trichloroacetic acid-induced liver tumors in female B6C3F1 mice." Cancer Letters 108 (1996): 257-61.

- Tao L, S Yang, M Xie, PM Kramer, and MA Pereira. "Hypomethylation and overexpression of c-jun and c-myc protooncogenes and increased DNA methyltransferase activity in dichloroacetic and trichloroacetic acid-promoted mouse liver tumors." Cancer Lett 158 (2000): 185-193.
- Tchantchou, Flaubert, Michael Graves, David Ashline, Amy Morin, Aurea Pimenta, Daniela Ortiz, Eugene Rogers, and Thomas B. Shea. "Increased transcription and activity of glutathione synthase in response to deficiencies in folate, vitamin E, and apolipoprotein E." Journal of Neuroscience Research 75 (2004): 508-15.
- Tiidus, Peter M., and Michael E. Houston. "Vitamin E Status and Response to Exercise Training." Sports Medicine 20 (1995): 12-23.
- Traber, M.G., 1999. Vitamin E. In: Modern Nutrition in Health and Disease. Ninth Edition. Baltimore: Williams & Wilkins, 1999, pp. 347-362.
- Walker, BE; J Kelleher; MF Dixon; MS Losowsky. "Vitamin E protection of the liver from paracetamol in the rats." Clin. Sci. Mol. Med. 47 (1974): 449-459.
- Wang, Xia, and Yong Lin. "Tumor necrosis factor and cancer, buddies or foes?" Acta Pharmacologica Sinica 29 (2008): 1275-288.
- Westerick, J. J., Mello, J. W., and Thomas, R. F. "The groundwater supply survey." J. Am. Water Works Assoc. 76 (1984): 52- 62.
- Zidenberg-Cherr, S., and C. Keen. "Influence of dietary manganese and vitamin e on adriamycin toxicity in mice." Toxicology Letters 30 (1986): 79-87.

