The induction of oxidative stress in the livers of mice following long-term exposure to the water chlorination by-products, dichloroacetate and trichloroacetate

Jacquelyn M. Cearfoss

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The Induction of Oxidative Stress in the Livers of Mice Following Long-Term Exposure
to the Water Chlorination By-Products, Dichloroacetate and Trichloroacetate

by

Jacquelyn M. Cearfoss

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Medicinal Chemistry

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May 2012
An Abstract of

The Induction of Oxidative Stress in the Livers of Mice Following Long-Term Exposure to the Water Chlorination By-Products, Dichloroacetate and Trichloroacetate

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Dichloroacetate (DCA) and Trichloroacetate (TCA) are known to be hepatotoxic and hepatocarcinogenic in rodents. DCA- and TCA-induced oxidative stress (OS) was hypothesized as a mechanism for the long-term hepatotoxicity of the compounds and was tested in the livers of B6C3F1 mice following subacute (4W) and subchronic (13W) treatment with doses ranging between non hepatotoxic and those producing maximal hepatotoxicity/hepatocarcinogenicity. The livers were assayed for the production of superoxide anion (SA), lipid peroxidation (LP) and DNA damage, as well as for the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and glutathione (GSH) levels. In general, the results indicated dose- and time-dependent increases in SA, LP and DNA damage, in response to both compounds, but hepatomegaly was observed after 13W treatment with 410 mg DCA/kg/day, and was associated with significant declines in those biomarkers. Increases in SOD, CAT and GSH-Px activities were observed at doses of 154-410 mg DCA/kg/day after 13W of treatment, but were unchanged or suppressed at the other doses and treatment periods.
TCA treatment, however, increased CAT and SOD activities but suppressed GSH-Px activity. Significant reduction in GSH level was only observed with 77-154 mg DCA/kg/day following 13W, with no effects observed with other treatments by either compound. In conclusion, DCA and TCA induced OS and modified antioxidant defenses at doses lower than those previously reported as hepatotoxic/hepatocarcinogenic, and at periods earlier than previously reported for the production of those effects. Vitamin E is a potent antioxidant, and DCA- and TCA-induced OS was assessed in the hepatic tissues of mice treated with the compounds, subchronically, by oral gavage, and fed either a standard (Std) diet or a diet without vitamin supplementation (Low-E). The livers of the Low-E diet groups demonstrated significant decreases in the biomarkers of OS, including production of SA, LP and DNA damage, and significant increases in antioxidant defense mechanisms as compared with the Std diet groups. It is concluded that vitamin E supplementation to the diet compromises the natural antioxidant defense mechanisms of the livers, which proved to be more effective than the vitamin itself.
Dedication

To two future scientists, my niece, Maeve Grace, and my goddaughter, Maya Kate.
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I thank all of my loved ones who cannot be here; I can feel your pride – Sláinte!

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Chapter 1: INTRODUCTION

1.1 Water Chlorination

Starting in the 1900s, and in an effort to decrease the incidence of waterborne pathogens, chlorine was introduced as a drinking water disinfectant (Miller and Uden 1983; Bull et al. 1995; Morris 1995). Investigation into water disinfection by-products linked to water chlorination began after chloroform, a known carcinogen, was found in the drinking water (Komulainen 2004). Several of the water disinfection by-products are formed following a chemical reaction between chlorine and organic matter, such as humic acid, located on surface water (Miller and Uden 1983; Bull et al. 1995; IARC 2004b; IARC 2004a). This led to the Safe Water Drinking Act (1977) (Boorman et al. 1999) to control the risk of exposure to the disinfection by-products found in the drinking water. Exposure to the disinfection by-products following chlorination of drinking water has been linked to increased incidences of bladder, esophageal and rectal cancers (McGeehin et al. 1993; Vena et al. 1993; Morris 1995; Tao et al. 1999).

1.2 Dichloroacetate and Trichloroacetate

Dichloroacetic Acid (DCA) and Trichloroacetic Acid (TCA) are two examples of disinfection by-products found in the chlorinated drinking water. These compounds have been found in the municipal water supplies at concentrations ranging between 30 to 160
µg/L (Uden and Miller 1983; IARC 2004b; IARC 2004a). DCA and TCA can also be formed *in vivo* following the metabolism of industrial solvents such as trichloroethylene and tetrachloroethylene (IARC 2004b; IARC 2004a). In 1998, the World Health Organization (WHO) established exposure guidelines to DCA and TCA at 50 µg/L and 100 µg/L, respectively (IARC 2004b; IARC 2004a).

The structures of dichloroacetate and trichloroacetate are shown below:

DCA has been used for the treatment of mitochondrial encephalomyelopathy associated with lactic acidosis (MELAS) in humans, and also for Huntington’s disease in mice (Andreassen et al. 2001; Mori et al. 2004). TCA has been used for the treatment of warts, dermal peeling and as an analytical agent (IARC 2004b).

Studies on DCA and TCA in rodents have found them to differ in their toxicokinetics. While DCA has a short plasma half-life and is highly metabolized *in vivo*, TCA was found to have a longer half life and is metabolized to a lesser extent. When rodents were administered [¹⁴C] DCA or [¹⁴C] TCA in the drinking water, the plasma half-life of DCA was reported as 0.6 to 1.6 hr, while that of TCA was 6 hr (Larson and Bull 1992). Also, half of a given dose of TCA was found to be excreted unchanged in the urine, while only 2% of a DCA dose was recovered unchanged in the urine (Larson and Bull 1992). However, the extent of DCA elimination is lowered with repeated dosing (James et al. 1998).
DCA is metabolized by the enzyme glutathione-S-transferase zeta (GSTZ1-1), requiring glutathione, and forming glyoxylic, oxalic and glycolic acids that are excreted in the urine (James et al. 1998; IARC 2004a). DCA inhibits its own metabolism by inactivating the enzyme GSTZ1-1 (Barton et al. 1999; IARC 2004a). This can occur when a chloride is removed from DCA to form $\text{S-}(\alpha\text{-chloro(carboxymethyl)}\text{GSH;}$ then this adduct either reacts with a cysteine residue modifying the enzyme, or can react with water to form glyoxylic acid and glutathione (Barton et al. 1999; IARC 2004a). DCA can also undergo reductive dechlorination by cytochrome P450 to form chlorinated acetates (Larson and Bull 1992). The dechlorination pathways lead to the production of either a monochloroacetyl radical, leading to the formation of monochloroacetic acid (MCA), or producing a monochloroacetyl peroxy radical which can form oxalate and glycolate (Larson and Bull 1992). DCA has also been found to be a metabolite of TCA. TCA undergoes reductive dechlorination catalyzed by cytochrome P450 producing a dichloroacetyl free radical (Larson and Bull 1992). The dichloroacetyl radical may abstract a hydrogen atom to form DCA, or it may react with oxygen to form a hydroperoxyl radical (Larson and Bull 1992; IARC 2004b).

1.3 Long-term Toxicity of Dichloroacetic Acid (DCA) and Trichloroacetic Acid (TCA)

Long term in vivo toxicity studies in rodents have found DCA and TCA to be hepatotoxic and hepatocarcinogenic. Herren-Freund et al. (1987) described DCA and TCA as complete hepatocarcinogens in male B6C3F1 mice. The studies involved 61 weeks of exposure to a dose of 5 g DCA or TCA per liter, which was administered in the
drinking water and resulted in the development of hepatocellular carcinomas and adenomas that were not initiated by ethylnitrosourea (ENU). Additionally, hepatocellular carcinomas were increased in ENU-initiated mice treated with either 2 or 5 g DCA or TCA per liter (Herren-Freund et al. 1987).

DeAngelo et al. (1991) treated B6C3F1 mice with DCA concentrations in the drinking water that were ranging between 0.05-5 g DCA/L for either 60 or 75 weeks, and found hepatic hyperplastic nodules and hepatocellular adenomas and carcinomas to be significantly increased in response to concentrations ranging between 3-5 g/L, as compared with the control (DeAngelo et al. 1991). The studies also found that concentrations of 0.05, 0.5, 3.5 and 5 g DCA/L were equivalent to 7.6, 77, 410, and 486 mg of DCA kg\(^{-1}\) day\(^{-1}\), respectively, based on time-weighted mean daily doses. They have also characterized the dose of 7.6 mg/kg/day as a nonhepatotoxic/hepatocarcinogenic dose, 77 mg/kg/day as the threshold dose for carcinogenicity, and 410-486 mg/kg/day as doses producing maximal hepatotoxicity/hepatocarcinogenicity (DeAngelo et al. 1991). A follow-up study on the same strain of mice, using doses ranging between 0.05 to 3.5 g DCA/L for 90-100 weeks has demonstrated a dose-dependent increase in DCA-induced hepatotoxicity (DeAngelo et al. 1999). Daniel et al. (1992) have also demonstrated increased liver weight and induction of hepatocellular necrosis, carcinomas and adenomas in male B6C3F1 mice treated with 0.5 g DCA/L in the drinking water for 104 weeks. Male and female B6C3F1 mice exposed to either 1 or 2 g/L of DCA or TCA in the drinking water for 52 weeks developed dose-dependent increases in final liver weight and final liver to body weight ratios (Bull et al. 1990). The studies also demonstrated significant increases in the hepatic lesions in response to the compounds when compared
with the control. DCA or TCA (2.0, 6.67, or 20.0 mmol/L) administered to female B6C3F1 mice in the drinking water for 360 or 576 days were also found to produce hepatocellular adenomas and carcinomas and altered hepatocytes (Pereira 1996). Increased liver and kidney organ to body weight ratios and increased hepatic β-oxidation activity were reported in Sprague-Dawley rats treated with either DCA (500 or 5000 ppm) or TCA (5000 ppm) in the drinking water for 90 days (Mather et al. 1990). Studies on rats have also found DCA to be hepatocarcinogenic at chronic doses of 0.5 g/L or 1.6 g/L that resulted in increased hepatocellular carcinomas and adenomas (DeAngelo et al. 1996).

### 1.4 Oxidative Stress

Oxidative stress (OS) is a result of cellular imbalance between the oxidant (reactive oxygen species (ROS) formation) and the antioxidant defense mechanisms (Machlin and Bendich 1987; Gutteridge 1995; Cadenas 1997). OS can result in cellular damage or death and is a contributing factor to several disease states (Aruoma 1999). ROS are produced endogenously during the metabolism of molecular oxygen via the mitochondrial respiratory chain. They can be also produced during the process of metabolism of xenobiotics and foreign substances, and as byproducts of some enzyme-catalyzed reactions (Bellomo 1991; Hayes and McLellan 1999). ROS can also play a role in cellular senescence, cellular growth and in apoptosis (Finkel 2003).

Hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^-$), (SA), and the hydroxyl radical (OH) are examples of ROS, and increases in their concentrations can be indicative of
OS. These ROS are commonly produced during a one-, two- or three-electron reduction of molecular oxygen (Davies 1995; Dreher and Junod 1996), as shown below.

SA is the first ROS produced in that process and is a source for the generation of \( \text{H}_2\text{O}_2 \) (Rice-Evans et al. 1995). SA has the ability to reduce iron (1), which allows hydrogen peroxide to react with the reduced form (\( \text{Fe}^{2+} \)) to make the hydroxyl radical via the Fenton reaction (2) (Halliwell and Gutteridge 1984; Ingold et al. 1993). Reactions (1) and (2) are demonstrated below:

\[
\begin{align*}
\text{O}_2^\cdot + \text{Fe}^{3+} &\rightarrow \text{O}_2 + \text{Fe}^{2+} & \quad (1) \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 &\rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- & \quad (2)
\end{align*}
\]

The hydroxyl radical is highly potent due to its’ short half-life, and therefore, can react quickly upon formation with surrounding biological molecules (Halliwell and Gutteridge 1984; Scandalios 1993).

Biomarkers of oxidative tissue damage include DNA damage and lipid peroxidation (LP). DNA damage can include single strand breaks (SSBs), base pair mutations, alkali labile sites and insufficient DNA repair mechanisms (Aruoma 1999; Waris and Ahsan 2006). LP, the breakdown of free fatty acids or cellular membranes, usually starts by an initiation reaction in which a proton is abstracted and a carbon radical is formed on the polyunsaturated fatty acid chain (Halliwell and Gutteridge 1984;
Gutteridge 1995). The carbon radical undergoes rearrangement to form a conjugated diene, and after reacting with oxygen, a hydroperoxy radical will be formed (Halliwell and Gutteridge 1984). Hydroperoxy radicals are able to abstract protons from surrounding lipid molecules, leading to propagation of lipid peroxidation (Halliwell and Gutteridge 1984).

ROS production is controlled in the cell by both enzymatic and non-enzymatic antioxidant defense mechanisms. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), are examples of the enzymatic defense mechanisms, and vitamin E, vitamin C and glutathione are examples of the non-enzymatic mechanisms, also known as scavengers (Bellomo 1991). SOD converts SA into hydrogen peroxide via reaction 3, and in turn, hydrogen peroxide is detoxified by CAT and GSH-Px as shown in reactions 4 and 5, respectively (McCord and Fridovich 1969; Scandalios 1993).

\[
\text{O}_2^+ + \text{O}_2^+ + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (3)
\]

\[
2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \quad (4)
\]

\[
\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + 2\text{GSSG} \quad (5)
\]

SOD antioxidant enzyme activity is diffusion-limited (Fridovich 1998) and different types of SOD enzymes are named based on the metal ion that is located in the active site. Copper or zinc SOD (Cu/ZnSOD) is a homodimeric enzyme located in the cytosol, in which copper is necessary for catalysis and zinc is present for structural stability (Scandalios 1993; Fridovich 1998; Aruoma 1999). Iron SOD (FeSOD) is another dimeric enzyme that is found in prokaryotes (Scandalios 1993). MnSOD is found in mitochondria and prokaryotes in tetrameric and dimeric forms, respectively, and contains
one Mn per subunit (Scandalios 1993; Fridovich 1998). While Cu/ZnSOD and FeSOD can be irreversibly inhibited by hydrogen peroxide, MnSOD is not affected by hydrogen peroxide (Scandalios 1993). MnSOD gene expression is regulated by two transcription factors, Sp1 and p53, that can bind to the MnSOD promoter gene (Dhar et al. 2011), and studies have reported changes in MnSOD activities in cancer cells, including hepatocellular carcinoma cell lines (Yang et al. 2005; Dhar et al. 2011).

CAT, a tetrameric enzyme, is located mainly in the peroxisome but also in the cytosol (Deisseroth and Dounce 1970). CAT contains four hematin prosthetic groups necessary for activity as well as multiple sulfhydryl and disulfide groups that are necessary for enzyme stabilization, but do not participate in the reduction of hydrogen peroxide (Deisseroth and Dounce 1970). CAT can decompose hydrogen peroxide catalytically forming water, or peroxidatively, which usually involves alkyl peroxides, by utilizing some metabolites as hydrogen donors for hydrogen peroxide reduction (Singh 1996). Whether CAT is reducing peroxides catalytically or peroxidatively, the initial event involves formation of a complex between hydrogen peroxide and the iron of the hematin prosthetic group (Deisseroth and Dounce 1970). During the catalytic process, the iron present is not reduced, i.e. it remains in the trivalent state, and the iron-hydrogen peroxide complex will react with another hydrogen peroxide molecule, which is the hydrogen donor, to form water as a product (Deisseroth and Dounce 1970). Experimental data have indicated that although a two-electron transfer may occur, the detoxification of hydrogen peroxide by CAT does not involve a free-radical intermediate (Deisseroth and Dounce 1970). SOD increases the protective effect of CAT because SA, which can
reduce the iron present in the active site, is a possible inhibitor of the antioxidant enzyme, catalase (Kono and Fridovich 1982).

GSH-Px requires selenium, present as selenocysteine at the active site, to participate in redox chemistry for catalysis (Flohe et al. 1973; Aruoma 1999). This enzyme is a tetramer that contains one selenium per subunit (Flohe et al. 1973). GSH-Px is located in the cytosol and mitochondria and utilizes reduced glutathione to convert hydrogen peroxide to two water molecules (Hayes and McLellan 1999; Singh 1996). In addition to detoxification, GSH-Px has the ability to inhibit the propagation of LP by reducing lipid hydroperoxides to alcohols (Flohe et al. 1973; Zimmermann et al. 1973; Burton et al. 1985). Glutathione is a tripeptide thiol and exists in either the reduced (GSH) or oxidized (GSSG) forms in the cell (Sies 1999). Glutathione provides defense against OS by scavenging free radicals and assisting in hydrogen peroxide reduction by GSH-Px (Hayes and McLellan 1999; Mari et al. 2009). When GSH-Px detoxifies hydrogen peroxide, a selenic acid (Se-OH) is formed at the active site of GSH-Px (Lubos et al. 2011). Reduced GSH is necessary for the reduction of Se-OH, which results in the formation of another intermediate (Se-SG) (Lubos et al. 2011). Oxidized glutathione (GSSG) is produced when another GSH molecule enters the GSH-Px active site to reduce the Se-SG bond and restore the GSH-Px active site (Lubos et al. 2011). Oxidized glutathione is reduced back to GSH by the enzyme glutathione reductase (GR) (Anderson 1985; Lubos et al. 2011).

Vitamin E is a lipid soluble, chain-breaking antioxidant that is comprised of 4 tocopherols and 4 tocotrienols: α, β, γ, and δ analogues (Burton et al. 1985; Gutteridge 1995; Schneider 2005; Brigelius-Flohe 2009). While the tocopherol and tocotrienol
families differ in the side chain attached to the chromanol ring, the analogues differ in the position and the number of methyl groups attached to the aromatic ring (Rimbach et al. 2002; Schneider 2005).

The most retained and active version of vitamin E in vivo is \( \alpha \)-tocopherol because the phenol is surrounded by three methyl (–CH\(_3\)) moieties (Ricciarelli et al. 2001), as seen below.

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 \\
\text{HO} & \quad \text{CH}_3 \\
\text{H}_3C & \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{CH}_3 \\
\end{align*}
\]

\((2R, 4’R, 8’R) \alpha \)-tocopherol

The \( \alpha \)-tocopherol transfer protein (\( \alpha \)-TTP) is responsible for facilitating the secretion of \( \alpha \)-tocopherol from the hepatic chylomicron remnants into the very low density lipoprotein (VLDL), which in turn secretes \( \alpha \)-tocopherol into the high density lipoprotein (HDL) and the low density lipoprotein (LDL) in the liver (Schneider 2005). The main dietary sources of vitamin E are plant foods, such as olive oil and sunflower seeds (Schneider 2005). Vitamin E deficiency was shown to result in neurologic lesions in rats, muscular dystrophy in mice and fat malabsorption and ataxia in humans (Schneider 2005). Vitamin E is also known to be a potent antioxidant that is lipid soluble. The antioxidant activity of \( \alpha \)-tocopherol is due to the phenolic hydrogen atom that can be readily donated to the hydroperoxy radical, ending the propagation of LP, and forming a tocopheroxyl radical (Rimbach et al. 2002; Schneider 2005). The tocopheroxyl radical can be reduced back to tocopherol by vitamin C or ubiquinol (Schneider 2005). However,
the tocopheroxyl radical is able to react with a second hydroperoxy radical, forming two non-radical species, and ending the antioxidant ability of that α-tocopherol molecule (Schneider 2005). In addition to its antioxidant function, vitamin E has demonstrated abilities to inhibit or activate enzymes, as well as to influence cellular signaling cascades and gene regulation (Brigelius-Flohe 2009).

1.5 DCA- and TCA-induced OS following short term exposures:

DCA- and TCA-induced oxidative stress (OS) was hypothesized as a mechanism for the long-term hepatotoxicity of the compounds. *In vitro* toxicity studies were conducted in order to determine the ability of DCA and TCA to induce OS. When J774.A1 macrophage cells were exposed to different concentrations of DCA or TCA for 24, 36 or 60 hours, time- and concentration-dependent increases were reported in SA production, LDH release by the cells, and cellular death (Hassoun and Ray 2003). Further *in vitro* studies on DCA effects on J744A.1 cells have demonstrated significant increases in the activities of SOD, CAT and GSH-Px and significant reduction in GSH level in response to the compound (Hassoun and Mehta 2008). Other *in vitro* studies on J774A.1 cells have demonstrated significant reductions in the percent of cell death, LDH leakage and SA production when the cells were treated with DCA or TCA, combined with tumor necrosis factor alpha (TNF-α) antibodies or SOD (Hassoun and Kini 2004).

Acute and subacute *in vivo* toxicity studies in rodents have also demonstrated the induction of OS by DCA and TCA. Celik (2007) found increased serum enzyme activities, increased hepatic SOD and CAT activities, and increased malondialdehyde (MDA) formation in the livers of Sprague-Dawley rats given 2000 ppm TCA in the
drinking water for 50 days. Mice administered a single oral dose of 300 mg/kg of either DCA or TCA demonstrated significant increases in the production of SA, LP and DNA-SSBs in the livers, 6 and 12 hour following DCA treatment, and 12 hours following treatment with TCA (Hassoun and Dey 2008). Single oral doses of either DCA or TCA (30, 100 or 300 mg/kg) administered to mice were found to induce dose-dependent increases in hepatic LP production and DNA damage, measured as formation of 8-hydroxyguanosine (8-OHdG/dG) (Austin et al. 1996).

Nelson and Bull (1988) used an alkaline unwinding assay to measure hepatic DNA-SSBs in Sprague-Dawley rats and B6C3F1 mice that were treated with a single, oral dose of either trichloroethylene (TCE) or its metabolites, DCA and TCA. While only high doses of TCE were found to induce DNA damage in rats, DNA damage was induced in a dose-dependent manner by lower doses of the metabolites (Nelson and Bull 1988).

When male B6C3F1 mice were treated with a single oral dose of either DCA or TCA, DNA damage, as measured by the alkaline unwinding assay, was significantly higher at 1, 2 and 4 hours post-treatment, as compared with the control (Nelson et al. 1989). The study also showed that when B6C3F1 mice were treated with either DCA (500 mg/kg) or TCA (500 mg/kg) for ten days; the compounds resulted in the production of significantly higher final liver weights and liver weight:body weight ratios in the treated mice, as compared with the control. Furthermore, the study demonstrated similar abilities for DCA and TCA to induce hepatic peroxisome proliferation (PP), but DCA treated mice demonstrated lower peroxisome β-oxidation than that produced by TCA in the liver homogenates (Nelson et al. 1989).
PP was also demonstrated in the hepatic tissues of B6C3F1 mice and Sprague-Dawley rats exposed to 1-5 g DCA or TCA/L or 1-3 g monochloroacetic acid (MCA)/L in the drinking water for 14 days (DeAngelo et al. 1989). While treatment with DCA resulted in increased PP in the hepatic tissues of rats, TCA treatment resulted in a significantly greater increase in hepatic PP in mice (DeAngelo et al. 1989). Additionally, there was a dose-dependent decrease in final body weight in rats treated with the chloroacetic acids, and a dose-dependent increase in relative liver weights in mice treated with DCA or TCA, as compared with that of the controls (DeAngelo et al. 1989).

Laughter et. al (2004) studied the effects of TCE, TCA and DCA in wild- and peroxisome proliferator-activated receptor (PPAR)-α-null SV129 mice, and have found TCA not to produce any significant increases in liver to body weight ratios in either mouse strain, but DCA and TCE increased those ratios in the PPAR-α-null mice, and wild-type mice, respectively (Laughter et al. 2004).
Chapter 2: OBJECTIVES

2.1 The induction of OS by DCA and TCA in the livers of mice after subacute and subchronic treatments (Study I):

The objective of Study I was to determine the induction of OS in the hepatic tissues of mice treated with doses of DCA or TCA ranging from non hepatotoxic/hepatocarcinogenic to those producing maximal hepatotoxicity/hepatocarcinogenicity, in the long-term carcinogenicity studies, and to assess the role of this mechanism in the production of those effects.

2.2 The effects of a low vitamin E diet on the induction of oxidative stress by DCA and TCA in the livers of mice (Study II):

The objective of Study II was to investigate the role of the potent antioxidant, vitamin E, in the protection against DCA- and TCA-induced oxidative stress, by demonstrating that marginal vitamin E deficiency would increase the risk for DCA- and TCA-induced OS, i.e., cancer production.
Chapter 3: METHODS

3.1 Chemicals
All of the chemicals and reagents used for this study, including sodium dichloroacetate (DCA) and sodium trichloroacetate (TCA), were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA) and were at the highest grade available.

3.2 Animals, Treatments and Liver Collections

Animals:
Male B6C3F1 mice, purchased from Harlan Teklad (Indianapolis, IN), were used because previous studies have demonstrated the sensitivity of this strain to the acute and long-term toxic effects of both, DCA and TCA (Herren-Freund et al. 1987; DeAngelo et al. 1989; Bull et al. 1990). Mice were approximately 6 weeks old and allowed to acclimate for 3 days prior to the beginning of the treatment. The mice were caged at 21°C with a 12 hour light/dark cycle and had free access to food and water.
3.2.1 Treatment of animals to determine the induction of OS by DCA and TCA in the livers of mice after subacute and subchronic exposure (Study I):

Mice were maintained on a standard laboratory chow diet purchased from Harlan Teklad (Indianapolis, IN). They were randomly assigned into groups of 7 animals and were administered 7.7, 77, 154, or 410 mg/kg of DCA or TCA daily for either four weeks (4W), or thirteen weeks (13W) via oral gavage. Control mice were administered 5.0 mL/kg distilled water. DCA and TCA solutions were prepared in distilled water, and the pH of the solutions, including the distilled water used for the control group, was adjusted to 7.0 using sodium hydroxide solution. The doses used for the study were based on previous long-term studies that found DCA and TCA to be hepatotoxic and hepatocarcinogenic (Herren-Freund et al. 1987; Bull et al. 1990; DeAngelo et al. 1991; DeAngelo et al. 1999). DeAngelo et. al (1991) treated mice chronically with 0.05, 0.5, 3.5 and 5.0 g/L of DCA, and calculated the time-weighted mean daily doses that corresponded to those doses and found them to be equivalent to 7.6, 77, 410 and 486 mg/kg/day, respectively. Since these doses ranged from nonhepatotoxic/hepatocarcinogenic to those producing maximal hepatotoxicity and hepatocarcinogenicity, they were selected for the study.

3.2.2 Treatment of animals to determine the effects of a low vitamin E diet on the induction of OS by DCA and TCA in the livers of mice (Study II):

Mice were randomly assigned into two groups that were fed either a Standard Laboratory Chow Diet (Std diet) or a Custom Research Diet (NIH-31 Mod) (Low-E diet); both purchased from Harlan Teklad (Indianapolis, IN). The Std diet contained vitamin E
supplementation with estimated level of 90 IU/kg of total E content, while the Low-E diet was not supplemented with vitamin E; containing only vitamin E from natural ingredients at a level of 20-30 IU/kg (Yasunaga et al. 1982). While severe vitamin E deficiency can cause adverse health effects, such as muscular atrophy and neurotoxic effects (Walsh et al. 1992; Ricciarelli et al. 2001; Schneider 2005; Traber 2007), the Low-E diet was expected to place the animals on marginal vitamin deficiency with no appreciable health problems (personal communications with the nutrition specialist at Harlan Teklad). This was confirmed by the daily monitoring of the animals for any changes in diet consumption, activity and overall health that revealed no abnormalities when compared with the group receiving the Std diet. Mice in either diet group were subdivided into 3 groups; each containing 7 animals. The subgroups in each diet group were treated with either 5.0 mL/kg distilled water (Control), 77 mg/kg DCA or 77 mg/kg TCA, daily by oral gavage for 13 weeks. The pH of DCA, TCA and control solutions was adjusted to 7.0, using sodium hydroxide solution. DeAngelo et. al (1991) calculated the time-weighted mean daily doses, following a chronic exposure period of B6C3F1 mice to various DCA concentrations in the drinking water, and found 0.5 g/L to correspond to 77 mg/kg/day, which was also identified as the threshold dose for hepatocarcinogenicity. Results obtained following Study I in our lab also demonstrated production of significant increases in the biomarkers of oxidative stress and the antioxidant defense mechanisms following 13 weeks of exposure to 77 mg/kg/day of either DCA or TCA. However, those increases either plateau or underwent decline in response to the higher doses that corresponded to the carcinogenic ones (Hassoun et al. 2010; Hassoun and Cearfoss 2011).
Collection of the livers for Study I and II:

At the end of the testing period for each study, mice were sacrificed using carbon dioxide anesthesia followed by cervical dislocation. The livers were collected, weighed, divided into 4 parts and used for determining SA and LP production, DNA-SSBs, antioxidant enzyme activities, and total glutathione (GSH), as described below.

3.2.3 Homogenization of the hepatic tissues

In order to measure superoxide anion (SA) and lipid peroxidation (LP) production following study I and study II, a 10% (w/v) homogenate of one part of the liver was made in Tris-KCl buffer (0.05M Tris-HCl and 1.15% KCl); pH adjusted to 7.4.

To measure DNA single strand break (SSB) production, one part of the liver was homogenized with the buffer of White et al. (1981), containing 120mM KCl, 30mM NaCl, 0.3mM spermine HCl, 1.0 mM spermidine HCl, 0.25M sucrose, 4.0mM EDTA, 1mM EGTA, 15mM Tris-HCl, 1mM PMSF and 15mM 2-mercaptoethanol to produce a 25% homogenate. The homogenate was centrifuged at 3000 g for 15 min and the nuclear fraction was re-suspended in half the volume of the buffer that was originally used. The re-suspended nuclear fraction was used for the assay.

The antioxidant enzyme activities of the livers, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), were determined in a (20% w/v) homogenate prepared with a part of the liver in a sucrose buffer containing 0.32M sucrose, 1mmol/L ETDA, 10mmol/L Tris-HCl. The tubes of the homogenates were centrifuged at 3000 g for 30 minutes and the supernatant was used for the assay of the antioxidant enzyme activities (Jiang et al. 1998).
Total glutathione (GSH) levels were measured using the 5,5-dithiobis (2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling method of Anderson et al. (1985). One part of the liver was homogenized in 5% sulfosalicylic acid (5 vol/g tissue), centrifuged for 5 minutes at 2500 rpm, and the supernatant was collected and used for the assay.

### 3.3 Superoxide anion (SA) determination

The method of Babior et al. (1984) was used to measure SA production, which is based on the reduction by SA of ferricytochrome c to ferrocytochrome c. Fifty μL of the Tris-KCl liver homogenate was mixed with 1.5 mL of 45 nmol cytochrome c solution in phosphate buffered saline (PBS), pH 7.2. The reaction tubes were incubated for 15 min at 37°C and were then placed on ice to quench the reaction. The tubes were centrifuged at 3000 rpm for 15 minutes and the absorbance of the supernatant was measured at 550 nm, a specific peak for cytochrome c reduction (Babior 1984), using a μQuant™ spectrophotometer (BioTek, Winooski, VT). The absorbance values were converted to nmol of cytochrome c reduced using the extinction coefficient 2.1 x 104 M⁻¹ cm⁻¹.

### 3.4 Determination of lipid peroxidation (LP)

Lipid peroxidation production was measured using the thiobarbituric acid reactive substances (TBARS) method (Uchiyama and Mihara 1977; Halliwell and Chirico 1993). In a large test tube, 0.5 mL of the Tris-KCl liver homogenate, 3 mL of 1% phosphoric acid, 1 mL of 0.6% thiobarbituric acid were mixed; and 100 μL of butylated hydroxyanisol (30 mg/30 mL) was also added to limit the formation of additional
oxidation products during the heating process. The reaction tubes were heated in a water bath (100°C) for 45 minutes. After cooling to room temperature, 4 mL of n-butanol was added to each reaction tube to abstract the pink chromagen containing TBARS, and centrifuged at 3000 rpm for 10 minutes. The absorbance of the n-butanol layer was measured at 535 nm, using a µQuant™ spectrophotometer (BioTek, Winooski, VT). The absorbance values were converted to nmoles of TBARS formed, using the extinction coefficient $1.556 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

3.5 Determination DNA Single Strand Breaks (DNA-SSBs)

DNA-SSBs were measured in the suspension of the nuclear fractions according to the method of Wahba et al. (1988). The hepatic nuclear suspensions (0.1 mL) were layered onto 5.0 μm SMWP Millipore filters (Millipore Corporation, Bedford, MA). Using a monostat cassette pump, the nuclear fractions were lysed at a speed of 200 μL min$^{-1}$ for 20 minutes with a lysing solution (2% (w/v) sodium dodecyl sulfate (SDS) and 25 mM EDTA, pH 10.3, adjusted with NaOH) in order to separate any DNA-protein interactions (Kohn et al. 1976). The nuclear fractions were then eluted at a rate of 100 μL min$^{-1}$ with an elution buffer containing 0.1% (w/v) SDS and 20 mM EDTA, pH 12.3, adjusted with the disodium salt of tetraethyl ammonium hydroxide to allow the separation of the strand breaks. Seven fractions of the eluate of the samples, 3 mL each, were collected using an Isco fraction collector (Isco, Lincoln, NE). Three mL of elution buffer were also added to 0.1 mL of the nuclear fraction of each sample and were treated like any fraction for the rest of the procedure, in order to determine the total amount of DNA in each sample mounted on the filter. DNA was precipitated by adding 0.1 mL of bovine
serum albumin (2.5 mg/mL BSA) and 1 mL of 40% trichloroacetic acid (TCA) to each fraction. The fractions were incubated at 4°C overnight and then centrifuged at 3000 rpm for 15 minutes. Each pellet was washed using 3.0 mL of a 36:1 Ethanol:HCl solution. The tubes were centrifuged at 3000 g for 10 minutes and the supernatant was discarded and the pellets were allowed to dry overnight in a fume hood. DNA-SSBs were measured in the pellets by adding 100 μL of 3,5-diaminobenzoic acid (270 mg/mL DABA) to each tube and incubating at 70°C for 35 minutes, after which 3 mL of 1M HCl was added to each tube and the fluorescence was measured at excitation 436 nm and emission 521 nm in a Shimadzu RF 5000U spectrofluorometer (Kyoto, Japan). The elution rate constant (k) was determined by plotting the log10 of the %DNA remaining on the filter after each fraction collection, against the cumulative volume of the eluate, where k = -2.3 x slope of this plot.

3.6 Determination of superoxide dismutase (SOD) activity

SOD activity was determined according to the method of Marklund and Marklund (1974), by measuring the inhibition of pyrogallol (1,2,3-benzenetriol) autoxidation. In short, a reaction mixture containing 250 μL of 2mM pyrogallol, 200 μL of the supernatant, and 750 μL of tricacodylic buffer, containing 50mM Tris-HCl, 50mM cacodylic acid, and 1mM EDTA, were combined in a test tube. The change in absorbance was measured for 3 minutes at 420 nm, using a Genesys™ 20 spectrophotometer (Thermo Scientific, Madison, WI). One unit of SOD is equivalent to the amount of SOD required for the inhibition of 50% pyrogallol autoxidation.
3.7 Determination of catalase (CAT) activity

CAT activity was measured according to the method of Cohen et al. (1970). Standard and blank reaction tubes were run simultaneously with the sample reaction tubes. The sample, standard and blank reaction tubes contained 100 µL of supernatant, 1.1 mL of distilled water, and 100 µL sucrose buffer, respectively. One mL of cold H₂O₂ (6mM) was added to the blank and sample tubes only. The tubes were incubated on ice for exactly 3 minutes, after which 200 µL of 6N H₂SO₄ was added in order to stop the reaction (Cohen et al. 1970). An excess (1.4 mL) of 0.002M KMnO₄ was added to each tube, and after reacting with the H₂O₂ present, the change in absorbance of the remaining KMnO₄ was measured for 3 minutes at 480 nm, using a Genesys™ 20 spectrophotometer (Thermo Scientific, Madison, WI). One unit of Enzyme equals k/0.00693, where k equals log [(S₀/S₂) x (2.3/t)]. S₀ is equivalent to the difference in the absorbance of the standard and the blank, S₂ is equivalent to the difference in the absorbance of the Standard and the Sample, and t is equal to the time of the reaction (3 minutes).

3.8 Determination of glutathione peroxidase (GSH-Px) activity

GSH-Px activity was measured indirectly by determining the oxidation of NADPH by the enzyme glutathione reductase (Lawrence and Burk 1976). A reaction mixture was prepared in PBS containing 1mM EDTA, 1mM sodium azide, 0.2mM NADPH and 1mM glutathione. One hundred µL of the supernatant sample, 700 µL of the reaction mixture and 100 µL of glutathione reductase (10 Units/mL) were combined in a reaction tube and incubated for 5 minutes at room temperature. Then, 100 µL of H₂O₂ (0.25mM) was added to each tube and the change in absorbance was recorded for 3
minutes at 340 nm, using a Genesys™ 20 spectrophotometer (Thermo Scientific, Madison, WI). The change in absorbance was converted to nmoles of NADPH oxidized per minute using an extinction coefficient of $6.22 \times 10^3$ L mol$^{-1}$ cm$^{-1}$.

### 3.9 Determination of glutathione (GSH)

Total GSH levels, the sum of the oxidized and reduced forms of glutathione, were measured using the DTNB-GSSG Reductase Recycling Method of Anderson (1985). The reduced form of glutathione is oxidized by 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) to form GSSG and 5-thio-2-nitrobenzoic acid (TNB) (Anderson 1985). The assay required a stock buffer, containing 143mM dibasic sodium phosphate and 6.3mM Na$_4$EDTA, and a daily buffer, containing 0.248 mg NADPH/mL of stock buffer. The assay tubes contained 700 μL of daily buffer, 100 μL of 6mM (DTNB), 175 μL distilled water and 25 μL of supernatant and were incubated at 30°C for 15 minutes. Then, 10 μL of glutathione reductase (266 U in stock buffer) was added to each tube to start the reaction. Glutathione reductase and NADPH reduce GSSG to GSH to continue the reaction (Anderson 1985). The change in absorbance was measured for 3 minutes at 412 nm, using a Genesys™ 20 spectrophotometer (Thermo Scientific, Madison, WI). A standard curve was prepared with reduced glutathione following the above procedure but replacing 25 μL of supernatant with a standard solution containing 1-4 nmol reduced glutathione.
3.10 Protein Determination

The amount of protein present in each sample was measured in the tissue homogenates using copper and the Folin reagent, and bovine serum albumin (BSA) was used as a standard (Lowry et al. 1951). The data obtained for SA, LP, SOD, GSH-PX and CAT were expressed per mg protein.

3.11 Statistical Analysis

Data were analyzed using Microsoft Excel data analysis package and GraphPad Prism®. Data are expressed as a means of 7 samples (animals) ± S.D. A one-way analysis of variance (ANOVA) was used to determine the statistical differences between groups followed by Tukey’s post-hoc test. A significance level of <0.05 was employed.
Chapter 4: RESULTS

4.1 The induction of OS by DCA and TCA in the livers of mice after subacute and subchronic treatments (Study I):

The effects of DCA and TCA on SA production in the livers of mice following 4W and 13W exposure periods are presented in figures 1A and 1B, respectively. Exposure to DCA produced significant increases in SA at all doses as compared with the corresponding controls of the 4W and the 13W treatment periods (fig. 1A). The increases in SA production in the livers of DCA-treated mice were dose-dependent following the 4W exposure period (fig. 1A). Hepatic SA production was significantly increased at the doses ranging between 7.7-154 mg DCA/kg/day following the 13W treatment period as compared with that of the corresponding 4W treatment groups (fig. 1A). At the highest 13W exposure dose of 410 mg DCA/kg/day, a decline in SA was observed when compared to both the immediately lower 13W dose and the same 4W dose (fig. 1A).

TCA-treated mice demonstrated significant increases in hepatic SA production at doses of 154 or 410 mg/kg/day for 4W as compared with that of the corresponding control (fig 1B). Following the 13W TCA treatment period, there were significant increases in hepatic SA production at doses ranging from 7.7 to 410 mg/kg/day, as compared with that of the corresponding control (fig 1B).
The production of LP in the livers of mice following exposure to varying doses of DCA and TCA is shown in figures 2A and 2B, respectively. Figure 2A shows that LP production in response to all of the tested doses in the two treatment periods was significantly higher in the livers of mice treated with DCA as compared with that of the corresponding controls in each period. However, Mice treated with DCA for 4W demonstrated a dose-dependent increase in hepatic LP production (fig. 2A), while the 13W exposure period demonstrated a dose-response relationship with DCA doses ranging between 7.7-154 mg/kg/day (fig. 2A). Mice treated for 13W with the highest dose, 410 mg DCA/kg/day, exhibited significantly decreased hepatic LP as compared with that of the immediately lower 13W dose and also when compared with that of the same 4W dose (fig. 2A). Figure 2B shows that hepatic LP production was significantly increased in the TCA-treated mice after the 4W treatment with doses ranging from 77 to 410 mg/kg/day, and for the 13 W treatments with doses ranging from 7.7-410 mg/kg/day. The observed increases in hepatic LP production after both treatment periods were dose-dependent.

Figures 3A and 3B show DNA-SSBs production in the livers of mice exposed to either DCA or TCA, respectively. There were significant increases in DNA-SSBs in the livers of mice treated with 77-410 mg DCA/kg/day for 4W, and likewise at all 13W DCA treatment doses as compared with those of the corresponding control groups (fig. 3A). There were no significant differences observed in hepatic DNA-SSBs production, following the comparison between either of the two highest 4W DCA treatment doses, or the 13W 77 and 154 mg/kg/day doses (fig. 3A). However, mice treated with 410 mg DCA/kg/day for 13W demonstrated a significant decrease in DNA-SSBs as compared with that of the immediately lower 13W dose (fig. 3A). Treatment with TCA resulted in
dose- and time-dependent increases in hepatic DNA-SSBs at doses ranging between 77-410 mg/kg/day (fig. 3B). However, a non significant difference was observed when comparing the highest TCA treatment doses of the two treatment periods (fig. 3B).

The DCA and TCA effects on the final liver weight (LW) and on the % LW/BW, at the end of each treatment period are shown in table 1. The LW and the %LW/BW were significantly increased for mice treated with 410 mg DCA/kg/day for 13W as compared with that of the corresponding control. There were no other significant differences in the final LW or %LW/BW in the treatment groups, as compared with that of the control mice.

Comparisons between DCA and TCA effects on the production of SA, LP and DNA-SSBs in the livers of mice treated for either 4W or 13W were made using a two-factor Analysis of Variance (ANOVA) without replication. Data obtained from the DCA and TCA dose-response curves for each biomarker (seven data points/dose) and for each time point were pooled together and then compared with each other. The p-values for such comparisons were determined and are presented in table 2. DCA produced more significant effects for each biomarker (p < 0.05) as compared with those effects produced by TCA within each treatment period, with the exception of LP production following the 13W treatment period, in which the comparison did not demonstrate significant difference in effects of the two compounds (p > 0.05).

The effects of exposure of mice to different doses of DCA and TCA on hepatic SOD activity are demonstrated in figures 4A and 4B, respectively. SOD activity was significantly suppressed in the livers of mice following 4W of exposure to DCA at all dose levels as compared with that of the corresponding control (fig. 4A). When mice
were treated with DCA for 13W, hepatic SOD activity was suppressed and significantly increased with doses of 7.7-77 mg/kg/day and 154-410 mg/kg/day, respectively, as compared with that of the 13W control (fig. 4A). Mice treated with TCA however, demonstrated dose- and time-dependent increases in the enzyme’s activity (fig. 4B).

The effects of DCA and TCA on CAT activity in the livers of mice are shown in figures 5A and 5B, respectively. CAT activity remained unchanged in the livers of DCA-treated mice in response to all of the tested doses following 4W treatment, and in response to doses of 7.7-77 mg DCA/kg/day following 13W treatment, as compared with those of the corresponding controls. Mice treated with either 154 or 410 mg DCA/kg/day for 13W showed significant increases in the enzyme’s activity as compared with that of the corresponding control (fig. 5A). Exposure to TCA resulted in a dose-dependent increase in hepatic CAT activity in response to doses of 77-410 mg/kg/day following the 4W treatment period, and in response to all of the tested doses, following the 13W treatment period (fig. 5B).

Figures 6A and 6B show GSH-Px activity in the livers of mice exposed to either DCA or TCA for either 4W or 13W. GSH-Px activity was not significantly changed in the livers of mice treated with DCA for 4W, as compared with that of the corresponding control (fig. 6A). While the DCA-treated mice exhibited suppression in hepatic GSH-Px activity in response to doses of 7.7 and 77 mg/kg/day in the 13W treatment period, mice treated with 154-410 mg DCA/kg/day for the same period exhibited significant increases in the hepatic enzyme’s activity, as compared with that of the corresponding control (fig. 6A). Exposure to TCA on the other hand resulted in significant suppressions in GSH-Px
activity in the livers of mice treated with any of the tested doses of the compound and at either treatment period when compared with the corresponding control (fig. 6B).

Total GSH levels in the livers of mice exposed to either DCA or TCA are shown in figures 7A and 7B, respectively. There were no changes in total GSH levels in the livers of mice exposed to the tested doses of DCA for 4W, as compared with that of the corresponding control (fig. 7A). While mice, exposed to 7.7 or 410 mg/kg/day of DCA for 13W demonstrated no change in total hepatic GSH levels, mice exposed to 77 and 154 mg of the compound/kg/day demonstrated significant suppression in total GSH, as compared with that of the corresponding control (fig. 7A). TCA-treated mice did not demonstrate any change in total hepatic GSH levels in response to any of the tested doses or the periods of treatments when compared with the corresponding controls (fig. 7B).

Supplemental figures, S1 and S2, present each biomarker of OS as % control following treatment with either 77 or 154 mg DCA or TCA/kg/day for each treatment period.

4.2 The effects of a low vitamin E diet on the induction of oxidative stress by DCA and TCA in the livers of mice (Study II):

Figure 8 shows the results of SA production as measured in the livers of mice fed either a Low-E diet or a Std diet and exposed to 77 mg/kg/day of either DCA or TCA for 13W. There were significant increases in SA production in the livers of mice treated with either DCA or TCA, regardless of the diet, as compared with those of the corresponding diet controls. Treatment with either DCA or TCA resulted in greater increases in hepatic SA production in mice fed the Std diet, as compared with those of the same treatments of
the Low-E diet groups. While treatment of the Low-E diet groups with DCA or TCA did not demonstrate a significant difference in SA production in the liver when compared with each other, mice treated with DCA and fed a Std diet showed a significantly greater increase in SA production as compared with that of the Std diet group treated with TCA.

The effects of either a Std or a Low-E diet on DCA- or TCA-induced LP production in the livers of mice are presented in figure 9. Treatment with DCA or TCA, regardless of the diet, resulted in significant increases in LP production as compared with the corresponding diet control. LP production was significantly higher in the livers of mice fed the Std diet and treated with either DCA or TCA, as compared with those of the corresponding Low-E diet treatment groups. While there was a significantly greater increase in LP production in the livers of mice treated with DCA and fed the Std diet when compared with that of mice fed the same diet and treated with TCA, there was no significant difference in hepatic LP production when treatments with the two compounds of the Low-E diet groups were compared.

The effects of either a Std or a Low-E diet on DCA- or TCA-induced DNA-SSBs production in the livers of mice are presented in figure 10. There were significant increases in DNA-SSBs in the livers of mice treated with DCA or TCA, regardless of the diet fed to the groups, as compared with those of the corresponding diet control groups. DNA-SSBs produced in the livers of mice treated with DCA and fed either diet were significantly higher than those of the TCA-treated in the corresponding diet groups. Furthermore, those mice fed the Std diet and treated with either DCA or TCA showed higher hepatic DNA-SSBs production as compared with that of the corresponding Low-E diet treated groups.
SOD activity was measured in the livers of mice fed either a Std or Low-E diet and treated with DCA or TCA and the results are shown in figure 11. Treatment with DCA resulted in suppression in hepatic SOD activity, regardless of the diet, as compared with that of the corresponding controls. However, SOD activities, in the livers of TCA-treated mice fed either the Std or Low-E diet, were significantly higher than those of the corresponding diet controls. Mice fed the Low-E diet, including the control group, demonstrated significant increases in hepatic SOD activity as compared with the corresponding Std diet treatment groups.

The effects of a Std or a Low-E diet on the hepatic CAT activity of mice treated with DCA and TCA are presented in figure 12. Treatment with DCA, regardless of the diet, did not result in any significant changes in hepatic CAT activity as compared with those of the corresponding control groups. On the contrary, mice fed either the Std or the Low-E diet and treated with TCA demonstrated increases in hepatic CAT activity when compared with those of the corresponding control diet groups. Also, CAT activity was induced to a significantly greater extent in the TCA-treated mice fed the Std diet, as compared with those mice fed the Low-E diet.

The effects of either a Std or Low-E diet on GSH-Px activity in the hepatic tissues of mice treated with either DCA or TCA are shown in figure 13. The livers of the control mice fed a Low-E diet demonstrated a higher GSH-Px activity than that of the Std-diet control. GSH-Px activities were significantly suppressed in the livers of mice fed either diet and treated with DCA or TCA, when compared with those of the corresponding diet controls.
The effects of either a Std or Low-E diet on the total glutathione (GSH) level in the livers of mice treated with DCA or TCA are shown in figure 14. The mice fed a Low-E diet and treated with DCA or TCA demonstrated significant increases in the total hepatic GSH levels as compared with that of either diet control; controls of the two diets demonstrated no significant difference in the level of that molecule. Mice fed the Std diet and treated with either DCA or TCA exhibited suppression and no change, respectively, in total GSH level, as compared with that of the corresponding control group.

Table 3 shows the final body weight (BW) on the day of sacrifice, liver weight (LW), and liver: body weight (LW/BW) ratio of the animals in each diet and treatment group. There were no significant differences in the final BW when comparing the DCA- and TCA-treated animals in each of the diet group with the corresponding controls, as well as when comparing the compounds-treated animals in the Std diet with the corresponding treatments in the Low-E diet groups. However, significant increases in LW and LW/BW were only revealed when DCA and TCA treatments in the Std diet groups were compared with the control of the Low-E diet group. Also, significant increases in those parameters were also revealed when comparing the DCA-treatment in the Std diet group with the corresponding treatment in the Low-E diet group.
Chapter 5: DISCUSSION

5.1 The induction of OS by DCA and TCA in the livers of mice after subacute and subchronic treatments (Study I):

DeAngelo et al. (1991) have previously reported a 136% increase in relative LW/BW in B6C3F1 mice, in response to a dose of 0.5 g/L of DCA administered in the drinking water for 60 weeks. The mean daily dose calculated for 0.5 g/L was 77 mg DCA/kg/day, and was identified as the threshold dose for DCA hepatocarcinogenicity (DeAngelo et al. 1991). For this study, the relative LW/BW ratio was increased 132% of the control in the DCA-treated mice in response to a dose of 410 mg DCA/kg/day following 13W of exposure. The increases reported in these two studies are equivalent and therefore, a dose of 410 mg DCA/kg/day can be considered as the threshold dose following a 13W treatment period. Hepatomegaly following chronic exposure to DCA resulted in altered foci of hepatocytes, focal necrosis and cancer production in the livers of B6C3F1 mice (Bull et al. 1990; Pereira 1996; DeAngelo et al. 1999) and DCA-induced hepatomegaly and focal necrotic lesions cause significantly higher and sustained cell proliferation (Sanchez and Bull 1993). Therefore, these cellular changes can be anticipated to occur at a dose of 410 mg DCA/kg/day when administered for 13W. Bull et al. (1990) reported increases in final LW and LW/BW in mice treated with 1 g/L (150
mg/kg/day) of TCA for 52 weeks. However, treatment with any of the doses of TCA did not result in significant increases in final LW following either treatment period for this study, which may suggest either a higher dose than 410 mg TCA/kg/day or a longer treatment period is required to demonstrate that effect.

Dose- and time-dependent increases in SA production were demonstrated following treatment with DCA and TCA. SA can be produced following the metabolism of xenobiotics via redox cycling or autoxidation reactions (Gibson et al. 1985; Bellomo 1991; Ingold et al. 1993). DCA and TCA undergo reductive dechlorination pathways during metabolism which may lead to increased SA production (Larson and Bull 1992). Following both treatment periods, DCA and TCA induced increases in LP and DNA-SSBs production for the tested doses in patterns similar to that of SA production, indicating a role of SA in the reported increases in LP and DNA-SSBs production.

LP production in response to TCA never did plateau, as was seen in the production of SA and DNA damage. The continuous increase in this biomarker may indicate its ultimate contribution to the TCA hepatotoxic effects expected to be produced with the extension of the treatment period, and may suggest it’s more significant contribution than SA and DNA damage to TCA-induced hepatotoxicity/hepatocarcinogenicity. DCA effects on various biomarkers of OS did reach maximal increases at doses of 154 and 410 mg/kg/day after 4W and 13W, respectively, with declines in those biomarkers observed at the highest dose, 410 mg DCA/kg/day, after the 13W treatment period. The observed plateau in the effects at the earlier time point together with the decline in various biomarkers of OS that were associated with hepatomegaly by a DCA dose of 410 mg/kg/day at a later time point,
indicate that those earlier maximal increases were sufficient to induce primary hepatic damage by the compound.

Except for LP production by DCA and TCA that was equally induced by the two compounds after 13 W of exposure, DCA produced significantly greater effects than TCA on each biomarker in the two treatment periods. In contrast to TCA, wherein 50% of a single dose is excreted unchanged, DCA was found to be highly metabolized in vivo in rats and mice, and only 2% of any single dose of DCA is recovered unchanged in the urine (Larson and Bull 1992; IARC 2004b; IARC 2004a). Since the production of free radicals during the metabolism of these compounds has been suggested as a factor in the generation of ROS by the compounds, fewer effects would be anticipated from a 50% dose of TCA, compared with a 98% dose of DCA.

SOD is an antioxidant enzyme that converts SA to hydrogen peroxide (Halliwell 1995; Johnson and Giulivi 2005). SOD activity was suppressed in the livers of DCA-treated mice for 4W and at the DCA doses of 7.7-77 following the 13W treatment period, indicating insignificant production of hydrogen peroxide in the livers of those treatment groups. On the other hand, SOD activity was increased at the DCA doses of 154-410 mg/kg/day for the 13W treatment period, thereby demonstrating significant production of hydrogen peroxide in response to these doses. MnSOD transcription is governed by the transcription factors Sp1 and p53, and was studied using a multistage skin carcinogenesis model that corresponded to decreased binding of Sp1 due to activation of p53 (Dhar et al. 2011). MnSOD expression was reported to decrease and increase between early and advanced stages of cancer, respectively (Dhar et al. 2011). This may explain the observed decreases and increases in SOD activity in response to DCA doses that were not
associated, or were associated with significant tumor production, respectively. Dose- and time-dependent increases in SOD activity were observed in the livers of the TCA-treated mice, indicating significant conversion of SA to hydrogen peroxide in response to the compound. Although the parallel increases in SA and SOD activities in response to TCA may indicate up-regulation of SOD transcription in response to SA overproduction, further studies to determine the enzyme amount may be required to confirm that.

Exposure of mice to varying doses of DCA for 4W induced LP production to a greater extent than was demonstrated following TCA exposure for the same time period. However, hepatic LP was increased to a similar extent by DCA and TCA following the 13W treatment period, indicating a slower induction of LP by TCA. This may be attributed to differences in the metabolism of the two compounds, where DCA undergoes metabolism and excretion at higher rates than TCA. Previous studies reported TCA metabolism to be catalyzed by cytochrome P-450 enzymes through a reductive dechlorination pathway forming a dichloroacetyl radical (Larson and Bull 1992). The radical species can abstract a proton from lipid molecules, forming DCA, and initiating LP (Larson and Bull 1992). The formation of a radical intermediate and a slower metabolism of TCA to DCA may have also contributed to the significantly higher induction of LP by TCA following the 13W treatment period, as compared with the effects seen after the 4W treatment period. The observation that SA production was not induced by TCA to the same extent as that for DCA after 13 W of exposure suggests that other mechanisms play a role in TCA-induced LP production, in addition to SA production. Hydrogen peroxide is another ROS that can contribute to LP production.
(Halliwell and Gutteridge 1984; Tong et al. 1998), and the dose- and time-dependent increases in SOD activity in the TCA treatment groups may confirm that suggestion.

Increased hydrogen peroxide can lead to increased production of more potent ROS, such as the hydroxyl radical. CAT and GSH-Px act in concert with SOD to detoxify hydrogen peroxide to water (Halliwell 1995; Tong et al. 1998). Insignificant production of hydrogen peroxide in the livers of the DCA-treated mice associated with suppressed SOD activity is confirmed by the indistinct changes or suppression observed in CAT and GSH-Px activities, respectively, in that group. Since the total SA produced is not completely converted to hydrogen peroxide, SA production can be considered as a main contributor to the induction of OS in the DCA-treated mice. While significant increases in SOD activity in TCA-treated mice were observed, these increases occurred along with increases and suppressions of CAT and GSH-Px activities, respectively. Therefore, the detoxification of hydrogen peroxide by CAT may have provided partial protection against this ROS effects in that group and also its partial contribution to the observed dose- and time-dependent increases in LP and DNA-SSBs production in that group. Also, DCA and TCA were found to act as peroxisome proliferators at doses falling in the range of those used in this study (DeAngelo et al. 1989; Nelson et al. 1989; Bull 2000), therefore the contribution of hydrogen peroxide via this source to DCA- and TCA-induced hepatotoxicity cannot be ignored.

Mice treated with the two highest doses of DCA (154-410 mg/kg/day) for 13W demonstrated significantly higher increases in the three tested antioxidant enzyme activities. An up-regulation of antioxidant activities was reported as a compensatory mechanism for normal cells in response to ROS-induced liver injuries as resulting from
assorted insults (Tsai et al. 2009; Dhar et al. 2011; Lubos et al. 2011). Previous studies have measured cellular modification, tumor formation and cancer production (Herren-Freund et al. 1987; Bull et al. 1990; DeAngelo et al. 1991; Daniel et al. 1992) at doses equivalent to those of the DCA’s that were associated with increased antioxidant enzyme activities in this study. Therefore, the observed increases in enzyme activities in response to those DCA treatments could have been associated with cellular modifications to maintain the survival of those cells. The increases in enzyme activities were also correlated with the observed decreases in the levels LP and DNA damage production at those doses, when compared with the immediately lower dose. Modification of antioxidant enzyme activities in response to cellular changes and modifications can be confirmed by the observation that the two lowest doses used in this study, 7.7 and 77 mg DCA/kg/day that correspond to a non-hepatocarcinogenic and threshold dose for carcinogenicity, respectively, (DeAngelo et al. 1991), were associated with suppression of those enzyme activities, rather than increases. It should also be remembered here, because of the proposed cellular modifications in those DCA-treatment groups, the values of various biomarkers of OS, including antioxidant enzyme activities, in their livers may no longer comparable to the control mice with normal hepatocytes.

Although total GSH levels remained unchanged in the livers of the TCA-treated mice, these levels were suppressed at DCA doses of 77 and 154 mg/kg/day following the 13W treatment period. DCA is metabolized and excreted at higher rates than TCA. In addition to the metabolism of DCA via a reductive dechlorination pathway catalyzed by cytochrome P-450 enzymes (Larson and Bull 1992), the compound has another metabolic pathway via glutathione S-transferase zeta-1 (GSTZ1-1) (Tong et al. 1998). However,
DCA is known to inhibit its own metabolism upon binding to GSTZ1-1, which uses GSH as a substrate, and a loss in enzyme activity was found to occur after repeated doses or prolonged drinking water exposures (Tong et al. 1998; IARC 2004a). Therefore, repeated exposure to DCA-induced GSTZ1-1 inhibition could have resulted in accumulation of electrophilic intermediates. Since GSH is known to detoxify free radicals other than ROS (Reed 1994), free radicals production via that pathway may have also contributed to GSH depletion in response to DCA. Although no changes in total GSH levels were observed with TCA treatments, there is still a possibility that the compound changes the ratio of the reduced/oxidized GSH, maintaining the total GSH level.

In conclusion, treatment of B6C3F1 mice with various doses of DCA and TCA resulted in dose- and time-dependent increases in all three biomarkers of OS, including SA, LP and DNA-SSBs production. These effects were induced at doses that have been previously identified as doses ranging between non hepatocarcinogenic to those producing maximal hepatocarcinogenicity, and at much earlier time points than was previously reported for the production of those effects. Therefore, the induction of these biomarkers of OS by DCA and TCA are considered as primary early events that can lead to the long-term hepatic effects. It is worth mentioning here that human risk assessment from exposure to various toxic chemicals is based on the doses that produce cancer in animals, which will be then converted to the No Observable Adverse Effect Level of the chemical after dividing it by an uncertainty or safety factor, which is usually 10 or several orders of magnitudes. These results may indicate that DCA and TCA risks may be underestimated if based on cancer production in animals, but may not be so if based on the earlier events, such as those determined in this study.
5.2 The effects of a low vitamin E diet on the induction of oxidative stress by DCA and TCA in the livers of mice (Study II):

Vitamin E is a strong antioxidant that protects against lipid peroxidation, maintains cellular membranes, and can act as a scavenger for free radicals. Since DCA and TCA were found to induce OS in mice, greater levels of OS were expected to be induced by the compounds in mice experiencing marginal vitamin E deficiency. However, the results of our studies contradicted that hypothesis.

Significantly greater SA was produced in the groups received a Std diet supplemented with vitamin E. In order not to reach toxic levels of vitamin E in the cell, the cellular level of the vitamin is regulated by the processes of metabolism and excretion (Mustacich et al. 2009), which involve up-regulation of gene expression of Phase I, II and III enzymes, such as cytochrome P450 and glutathione S-transferase (Kluth et al. 2005; Selman et al. 2008; Brigelius-Flohe 2009; Mustacich et al. 2009). SA and free radicals can be produced following the metabolism of xenobiotics (Hayes and McLellan 1999). Metabolism of DCA is catalyzed by glutathione S-transferase zeta (GSTZ1-1), and DCA and TCA can be metabolized via reductive dechlorination pathways catalyzed by the cytochrome P-450 enzymes, leading to the production of several free radicals that can contribute to the production of ROS by the compounds (Larson and Bull 1992; Stacpoole et al. 1998; Tong et al. 1998). Therefore, the process of vitamin E regulation in the Std diet groups may have contributed to the observed increases in SA production in those groups, as compared with the Low-E diet groups.

While previous studies have reported increases in LP production in the livers of rats and muscle tissue of calves following vitamin E deficiency (Masugi and Nakamura
our studies reported increases in this biomarker in mice fed the Std diet. It is well known that increases in the production of ROS can lead to increases in other biomarkers of OS, such as LP and DNA damage (Hayes and McLellan 1999; Waris and Ahsan 2006), and therefore, the observed increases in the latter two biomarkers in the Std diet groups are very well-correlated with the observed increases in SA production, in response to the compounds. The differences in the results reported by our studies and the studies of other investigators could be related to the severe vitamin E deficiency induced in the animals by the other studies, as compared with the marginal E deficiency induced in the animals by our studies. Also, the animals in the previous studies did not receive treatment with any compound that can invoke an oxidant response, thereby initiating an antioxidant response (Masugi and Nakamura 1976; Xu and Diplock 1983; Walsh et al. 1992). Increases in LP production in the Std diet groups can also be attributed to α-tocopherol-mediated peroxidation (TMP) that occurs primarily in the LDL particle containing bis-allylic methylene groups and molecules of α-tocopherol, ubiquinol-10 and oxygen (Schneider 2005). The process involves donation of a proton by α-tocopherol to provide an antioxidant defense, forming the α-tocopherol radical. Since LDL can only accommodate one radical at a time (James et al. 1998; Schneider 2005), this α-tocopherol radical has the opportunity to extract a proton from a nearby bis-allylic methylene, before being reduced via vitamin C or ubiquinol-10, resulting in the formation a fatty acid radical which can initiate LP (Rice-Evans et al. 1995; Ricciarelli et al. 2001; Schneider 2005). This may also be confirmed by the studies demonstrating decreased LDL auto-oxidation with decreased α-tocopherol concentrations (Culbertson et al. 2001). Although α-tocopherol is known as an antioxidant, it can also act as a pro-oxidant because it can
react with transition metal ions, converting the oxidized metal ions to reduced forms (Schneider 2005). Reduced transition metal ions can react with peroxides that are present, e.g. hydrogen peroxides, producing more potent ROS, such as the hydroxyl radical, and alkoxy radicals (Halliwell 1995; Schneider 2005). The pro-oxidant activity of α-tocopherol associated with possible formation of more potent ROS may further contribute to the observed increases in LP and DNA-SSBs production in the livers of the mice fed the Std diet.

Antioxidant enzyme activities, while usually suppressed in response to the production of high levels of ROS, were also found to undergo adaptive increases in response to certain smaller concentrations of those species, maintaining the prooxidant-antioxidant balance of the cell (Chow et al. 1973; Cabo et al. 2006). Superoxide dismutase (SOD) is an antioxidant enzyme that converts SA to hydrogen peroxide, and it also acts in concert with CAT and GSH-Px, where the latter two enzymes convert hydrogen peroxide to water (Burton et al. 1985). Hepatic SOD activity was suppressed in the Std and the Low-E diet groups treated with DCA, suggesting insignificant production of hydrogen peroxide in those groups. The non significant changes in CAT activity and the suppression of GSH-Px activity in those groups may also suggest insignificant detoxification of SA, and the contribution of this ROS to the observed increases in LP and DNA-SSBs production. However, SOD activities were significantly increased in the hepatic tissues of the Std and the Low-E diet groups treated with TCA, which were also associated with significant increases in CAT activities, but suppression of GSH-Px activities. This may suggest partial detoxification of SA in the TCA-treated groups and
also the possible contribution of hydrogen peroxide to the observed increases in LP and DNA damage production.

While vitamin E supplementation could have contributed to the observed levels of SA and its associated levels of LP and DNA damage in the control of the Std diet group, the increases in SOD and GSH-Px activities in the control of the Low-E diet group may have accounted for the leveling of SA, LP and DNA damage to those of the Std diet control. The increase in SOD activity in the Low-E diet group may reflect an adaptation mechanism to keep up with overproduction of SA (in the absence of vitamin E supplementation) in that group forming hydrogen peroxide that was simultaneously detoxified by GSH-Px. Also, GSH-Px was found to protect against LP by reducing organic hydroperoxides to oxidized glutathione and water (Chow et al. 1973; Hayes and McLellan 1999). Following meta-analysis of vitamin E human randomized trials, it was concluded that general supplementation with alimentary antioxidants will change normal cellular processes, and that supplementation with α-tocopherol can affect the absorption of the other analogues of vitamin E which may be more beneficial in the protection of OS (Hensley et al. 2000; Gee 2011); others have demonstrated an endogenous relationship between GSH-Px and vitamin E (Flohe et al. 1973; Machlin and Bendich 1987). Hence, all these may have collectively contributed to the observed levels of the various biomarkers of OS in the controls of the two diet groups.

GSH is an important antioxidant that serves as a substrate for GSH-Px and also has a potent scavenging ability for various free radicals (Hayes and McLellan 1999; Sies 1999). The observed increases in GSH levels in the Low-E diet groups treated with DCA or TCA can be viewed as an adaptation response to the lack of protection against ROS.
overproduction that was alternatively provided by vitamin E supplementation in the Std diet groups. Tchantchou et. al (2004), found that total GSH and glutathione synthetase levels were increased in mice fed a diet deficient in vitamin E. Also, Cabo et. al (2006) have demonstrated increased GSH levels in rats fed a vitamin E deficient diet. Increased glutathione synthetase levels was suggested to be an adaptive antioxidant response (Hayes and McLellan 1999); therefore, the observed increases in GSH may have been associated with increased GSH synthetase activity. Since GSH is a potent scavenger of free radicals and aids in the detoxification of hydrogen peroxide, the increases in this antioxidant molecule may have contributed to the lower levels of SA and its associated biomarkers LP and DNA damage in the Low-E diet groups treated with the compounds.

Exposure to DCA and TCA resulted in increases in the final LW and LW/BW of the Std diet treated animals, only when compared with the Low-E diet control group. An increase in LW can result from exposure to peroxisome proliferators, such as DCA and TCA (Maronpot et al. 2010), as well as from enzyme induction, especially the cytochrome P-450 enzymes (Kluth et al. 2005; Mustacich et al. 2009). Vitamin E deficiency has been reported to alter the concentrations of microRNA regulators in the livers of rats leading to decreases in the amounts of certain enzymes (Gaedicke et al. 2008). The decreases in the amount of certain hepatic enzymes in the Low-E diet groups may have resulted in decreases, albeit non-significant, in the liver weight of those groups when compared with the control of the Std diet groups. Also, DCA- and TCA-induced peroxisome proliferation may have contributed to some increases in the liver weights, but those increases were non-significant when compared with the corresponding diet control. However, when these two factors were added together in the process of statistical
comparisons, i.e., decreases due to decreased dietary vitamin E in the Low-E diet control and increases due to TCA and DCA treatment in the Std diet groups, they both contributed to the overall observed significant increases in the liver weights of the Std diet groups treated with the compounds, compared to the control of the Low-E diet group.

In conclusion, the observed increases in GSH, and GSH-Px and SOD activities and the decreases in LP and DNA-SSBs in response to DCA and TCA in mice fed the Low-E diet indicates a production of protective effects in response to that diet against the compounds-induced OS, i.e., although vitamin E supplementation to the diet may be essential to maintain various body and cellular functions, it can compromise the effectiveness of other natural antioxidants defense mechanisms. Additionally, the significant increases in the LW and LW/BW by the compounds as demonstrated in the Std diet groups, compared with the Low-E diet control group may indicate a possible increase in the compounds risk to induce hepatotoxicity when fed a Std diet. Although this conclusion can be interpreted as a recommendation for non supplementation of the vitamin to the diet, it does not advise removal of this vitamin from the diet, especially when the partial protection against the compounds-induced OS/ hepatotoxicity provided by non vitamin supplementation is weighed against the possible loss of other essential effects of the vitamin on different body and cellular functions.
Figure 1A:
SA production, determined as cytochrome c reduced min⁻¹ mg⁻¹ protein in the hepatic tissues of mice treated with DCA, 4 and 13 weeks after treatment. Each value is the mean of seven samples (seven animals) ± SD. The effects of different doses, including the control, were compared against each other in the same treatment period, as well as in the other period of treatment. Columns with non identical superscripts are significantly different (p < 0.05).
SA production, determined as cytochrome c reduced min⁻¹ mg⁻¹ protein in the hepatic tissues of mice treated with TCA, 4 and 13 weeks after treatment. Each value is the mean of seven samples (seven animals) ± SD. The effects of different doses, including the control, were compared against each other in the same treatment period, as well as in the other period of treatment. Columns with non-identical superscripts are significantly different (p < 0.05).
Figure 2A:
LP production, determined as nanomoles TBARS produced per mg of protein in the hepatic tissues of mice treated with DCA following 4 and 13 weeks of treatment. Each value is the mean of seven samples (seven animals) ± SD. The effects of different doses, including the control, were compared against each other in the same treatment period, as well as in the other period of treatment. Columns that do not share an identical superscript are significantly different (p < 0.05).
Figure 2B:
LP production, determined as nanomoles TBARS produced per mg of protein in the hepatic tissues of mice treated with TCA following 4 and 13 weeks of treatment. Each value is the mean of seven samples (seven animals) ± SD. The effects of different doses, including the control, were compared against each other in the same treatment period, as well as in the other period of treatment. Columns that do not share an identical superscript are significantly different (p < 0.05).
Figure 3A:
DNA-SSBs production, indicated by the elution rate constant \( k \), in the hepatic tissues of DCA-treated mice, following 4 and 13 weeks of treatment. Each value is the mean of seven samples (seven animals) ± SD. The effects of different doses, including the control, were compared against each other in the same treatment period, as well as in the other period of treatment. Columns with non identical superscripts are significantly different \( (p < 0.05) \).
Figure 3B:
DNA-SSBs production, indicated by the elution rate constant (k), in the hepatic tissues of TCA-treated mice, following 4 and 13 weeks of treatment. Each value is the mean of seven samples (seven animals) ± SD. The effects of different doses, including the control, were compared against each other in the same treatment period, as well as in the other period of treatment. Columns with non identical superscripts are significantly different (p < 0.05).
Figure 4A:
SOD activity determined in the hepatic tissues of mice treated with DCA, after 4 or 13 weeks of treatment. The effects of different doses (including the controls), and different lengths of treatment were compared. Columns with non identical superscripts are significantly different (p < 0.05).
Figure 4B:
SOD activity determined in the hepatic tissues of mice treated with TCA, after 4 or 13 weeks of treatment. The effects of different doses (including the controls), and different lengths of treatment were compared. Columns with non identical superscripts are significantly different (p < 0.05).
Figure 5A: Catalase activity determined in the hepatic tissues of mice treated with DCA following 4 or 13 weeks of treatment. The effects of different doses (including the controls), and different lengths of treatment were compared. Columns that do not share identical superscripts are significantly different (p < 0.05).
Figure 5B:
Catalase activity determined in the hepatic tissues of mice treated with TCA following 4 or 13 weeks of treatment. The effects of different doses (including the controls), and different lengths of treatment were compared. Columns that do not share identical superscripts are significantly different (p < 0.05).
Figure 6A:
Glutathione peroxidase activity in the hepatic tissues of mice treated with DCA following 4 and 13 weeks of treatment. The effects of different doses (including control), and different lengths of treatment were compared. Columns that do not share identical superscripts are significantly different (p < 0.05).
Figure 6B: Glutathione peroxidase activity in the hepatic tissues of mice treated with TCA following 4 and 13 weeks of treatment. The effects of different doses (including control), and different lengths of treatment were compared. Columns that do not share identical superscripts are significantly different (p < 0.05).
Figure 7A:
Total glutathione determined in the hepatic tissues of mice treated with DCA following 4 and 13 weeks of treatment. An asterisk (*) indicates significant difference when compared with the corresponding control (p < 0.05).
Figure 7B:
Total glutathione determined in the hepatic tissues of mice treated with TCA following 4 and 13 weeks of treatment. An asterisk (*) indicates significant difference when compared with the corresponding control (p < 0.05).
Figure 8:

SA production determined as cytochrome c reduced/min/mg protein in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly different (p<0.05).
**Figure 9:**
LP, indicated as TBARS production in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly (p<0.05).
Figure 10:
DNA SSBs production, indicated by the elution rate constant (k), in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly different (p<0.05).
Figure 11:
SOD activity (units/mg protein) determined in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly different (p<0.05).
**Figure 12:**
CAT activity (Units/ mg protein) determined in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly different (p<0.05).
Figure 12:
CAT activity (Units/mg protein) determined in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly different (p<0.05).

Figure 13:
GSH-Px activity determined as nmoles NADPH oxidized/min/mg protein in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly different (p<0.05).
Figure 14:
Total GSH level (nmoles/g tissue) determined in the livers of mice kept on either a Std, or Low-E diet and treated subchronically with DCA or TCA. Columns with non identical superscript are significantly different (p<0.05).
### Table 1:

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DCA and TCA effects on the liver weight (LW) and the % LW/BW at the end of the treatment periods. Each value is a mean of seven samples (animals) ± SD. An asterisk (*) denotes significantly different from the corresponding control (p < 0.05), using t-test.
Table 2:

P-values for comparisons between the effects of DCA and TCA on the production of SA, LP, and DNA-SSBs at each period of treatment, using two-factor ANOVA without replication, p > 0.05 is non-significant.

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<td></td>
<td>Final BW</td>
<td>LW</td>
<td>LW/BW</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>-----</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>Std</td>
<td>Low-E</td>
<td>Std</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>34.0 ± 2.1</td>
<td>33.6 ± 1.8</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td><strong>DCA</strong></td>
<td>34.4 ± 2.3</td>
<td>34.0 ± 2.3</td>
<td>1.9 ± 0.2&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>TCA</strong></td>
<td>35.3 ± 0.99</td>
<td>34.9 ± 2.5</td>
<td>2.0 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 3:**

Parameters, including body weight (BW), liver weight (L) and L:BW ratio were measured/calculated in groups of animals treated with DCA or TCA in the Low-E and Std diet groups. Values within each parameter that share an identical superscript are significantly different, p<0.05. Values within each parameter that are not labeled with any superscript did not demonstrate any significant differences when compared with each other.
Each biomarker of OS, including SA and LP production, DNA damage, antioxidant enzyme activities and total GSH levels, was calculated as % control following treatment with either 77 or 154 mg DCA/kg/day for each treatment period.
S2: Each biomarker of OS, including SA and LP production, DNA damage, antioxidant enzyme activities and total GSH levels, was calculated as % control following treatment with either 77 or 154 mg TCA/kg/day for each treatment period.
Chapter 6: REFERENCES


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