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The Effects of a Low Vitamin E Diet on Dichloroacetate- and Trichloroacetate-Induced Oxidative Stress in the Livers of Mice

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Abstract

Groups of mice were fed either a standard (Std) diet, or a diet not supplemented with vitamin E (Low-E) and were divided into three subgroups that were treated subchronically, by gavage, with water (control), DCA, or TCA. The livers of the animals were assayed for various biomarkers of oxidative stress (OS), antioxidant enzyme activities and total glutathione (GSH). In general, livers from the low-E diet group expressed lower levels of biomarkers of OS associated with greater increases in various antioxidant enzymes activities and glutathione when compared with the corresponding treatments in the Std diet group. These results suggest that vitamin E supplementation to the diet while essential to maintain certain body functions; it can compromise the effectiveness of the hepatic antioxidant enzymes and GSH resulting in an increase in DCA- and TCA-induced OS and a possible increase in the compounds-induced hepatotoxic/hepatocarcinogenic effects in mice.

INTRODUCTION

Dichloroacetate (DCA) and trichloroacetate (TCA) are by-products formed during the process of drinking water chlorination and were also found to be formed *in vivo* in humans and rodents by the metabolism of the widely used industrial solvents, trichloroethylene and tetrachloroethylene [1, 2]. The World Health Organization (WHO) established provisional guidelines for DCA and TCA concentrations in the drinking water of 50 and 100 µg /L, respectively [1, 2].

Studies on the long term toxicity of DCA and TCA in rodents have identified hepatotoxicity, especially hepatomegaly, and hepatocarcinogenicity as major effects. The compounds were found to be hepatocarcinogenic in male B6C3F1 mice following 61 weeks exposure to 2–5 g/L [3]. DeAngelo et al. [4] confirmed the hepatocarcinogenic effects of DCA in the same mouse strain exposed to doses ranging from 0.05 to 5 g/L for 60–75 weeks. A follow-up study with doses ranging between 0.05 to 3.5 g DCA/L administered to the same strain of mice for 90–100 weeks demonstrated dose-dependent increases in the hepatotoxicity and the increases in tumor incidences at doses > 0.5 g DCA/L [5]. Increased hepatoproliferative lesions and hepatomegaly were also seen in male B6C3F1 mice exposed to either 1 or 2 g/L of DCA or TCA in the drinking water for 52 week [6], and hepatocellular adenomas and carcinomas and altered hepatocytes were demonstrated in the livers of female B6C3F1 mice exposed to 2.0–20.0 mmol DCA or TCA /L for 360–576 days [7].

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DCA and TCA were found to induce oxidative stress (OS) in the hepatic tissues of mice following acute, subacute and subchronic exposure. Single oral doses of either DCA or TCA induced significant production of various biomarkers of OS in the hepatic tissues of B6C3F1 mice, including lipid peroxidation (LP), DNA damage, and superoxide anion (SA) [8–10]. Induction of the biomarkers of OS and modulation of various antioxidant enzyme activities were also demonstrated in the livers of B6C3F1 mice exposed to subacute and subchronic doses of DCA and TCA, and were suggested to be early contributing mechanisms to the later development of the hepatocarcinogenic/hepatotoxic effects of the compounds [11, 12]. Vitamin E is a strong chain-breaking, lipid soluble antioxidant molecule that supports biological membranes [13, 14]. Based on that significant biological role of the vitamin, we hypothesized that mice receiving a diet not supplemented with the vitamin would be at a greater risk for OS induction by DCA and TCA after long term exposure and that may eventually increase the compounds hepatotoxic/hepatocarcinogenic risks. However, the outcome of our studies contradicted that hypothesis.

METHODS

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.

Animals and Treatments

Experiments in this study were conducted on animals according to a protocol that was approved by the Institutional Animal Care and Use Committee of the University of Toledo, following the NIH guidelines. 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 [3, 6, 12, 15, 16]. 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. They were randomly assigned into two groups that were fed either a Standard Laboratory Chow Diet (Std diet) or Custom Research (NIH-31 Mod) (Low-E diet); both purchased from Harlan Teklad (Indianapolis, IN). The Std diet contained the regular 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 an approximate level of 20–30 IU/kg [17]. While vitamin E supplementation of the Std diet is believed to provide optimal immunopotentiality, growth, and reproduction in mice [17–19], and only severe vitamin E deficiency is known to induce myopathy [20–22], the Low-E diet was expected to place the animals on marginal E 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, body weight and overall health that revealed no abnormalities when compared with the group receiving the Std diet. Mice in either group were divided into 3 subgroups which were treated with either 5.0 ml/kg distilled water (Control), 77 mg/kg/day DCA or 77 mg/kg/day TCA, daily by gavage for 13 weeks. The pH of DCA and TCA solutions, as well as distilled water administered to the controls, was adjusted to 7.0 using sodium hydroxide solution. DeAngelo et al. [4] 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, a concentration that corresponded to the threshold dose for hepatocarcinogenicity, was equivalent to 77 mg/kg/day. Also, previous studies in our lab have demonstrated the induction of significant increases in the biomarkers of OS and modulation of antioxidant enzyme activities by a dose

of 77 mg DCA or TCA/kg/day, after 13 weeks of exposure, and that the observed increases were intermediate between other lower and higher, hepatotoxic doses [11, 12]. Therefore the dose was selected to allow observing any increases or decreases in response to the Low-E diet.

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

Sample preparation for the determination of SA and lipid LP production

To determine the levels of SA and LP in the hepatic tissues, 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.

Superoxide anion (SA) determination

The cytochrome c reduction method of Babior et al. [23] was used to measure SA production. 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 550nm, using a $\mu\text{Quant}^{\text{TM}}$ spectrophotometer (BioTek, Winooski, VT). The absorbance values were converted to nmoles of cytochrome c reduced/min, using the extinction coefficient $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Determination of lipid peroxidation (LP)

Lipid peroxidation production was measured using the thiobarbituric acid reactive substances (TBARS) method [24, 25]. 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. One hundred μL of butylated hydroxyanisole (30 mg/30 ml) was added to each tube 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 and centrifuged at 3000 rpm for 10 minutes for TBARS extraction. The absorbance of the n-butanol layer was measured at 535 nm, using a $\mu\text{Quant}^{\text{TM}}$ spectrophotometer (BioTek, Winooski, VT). The absorbance values were converted to nmoles of TBARS formed, using the extinction coefficient $1.556 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Determination DNA Single Strand Breaks (DNA SSBs)

Part of the liver from each animal was homogenized with the buffer of White et al. [26], 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 25% w/v 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. DNA SSBs were measured in the suspension of the nuclear fractions according to the method of Wahba et al. [27]. 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 $200 \mu\text{L min}^{-1}$ for 20 minutes with a lysing solution containing 2% (w/v) sodium dodecyl sulfate (SDS) and 25 mM EDTA, pH10.3, adjusted with NaOH. The nuclear fractions were then eluted at a rate of $100 \mu\text{L min}^{-1}$ with an elution buffer containing 0.1% (w/v) SDS and 20 mM EDTA, pH

12.3, adjusted with disodium salt of tetraethyl ammonium hydroxide. Seven fractions of the eluate of each sample, 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 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 3000g 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 $\log_{10}$ of the %DNA remaining on the filter after each fraction collection, against the cumulative volume of the eluate, where $k = -2.3 \times \text{slope of the plot}$.

Sample preparation for the determination of the antioxidant enzyme activities

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities were determined in a (20% w/v) homogenate prepared with part of the livers in sucrose buffer containing 0.32M sucrose, 1mmol/L EDTA, 10mmol/L Tris-HCl, according to the method of Jiang et al. [28]. The tubes were centrifuged at 3000 g for 30 minutes and the supernatant was used for the assay.

Determination of SOD activity

SOD activity was measured according to the method of Marklund and Marklund [29]. In short, a reaction mixture was prepared by mixing 200 µL of the supernatant with 750 µL of trisacodylic buffer containing 50mM Tris-HCl, 50mM cacodylic acid, 1mM EDTA, 250 of µL of 2mM pyrogallol 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 to inhibit 50% of pyrogallol autooxidation.

Determination of CAT activity

CAT activity was measured using the method of Cohen et al. [30]. A 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 and 1.4 ml of 0.002M KMnO₄ were added to each tube and the change in absorbance 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 $\log [(S_0/S_2) \times (2.3/t)]$. S₀ is equivalent to the difference in absorbances of the standard and the blank, S₂ is equivalent to the difference in the absorbances of the standard and sample, and t is equal to the time of reaction (3 minutes).

Determination of GSH-Px activity

GSH-Px activity was measured using the method of Lawrence and Burk [31]. 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 reaction mixture

and 100 μ L of glutathione reductase (10 units/ml) were combined in a reaction tube and incubated for 5 minutes at room temperature. Then, 100 μ L of H_2O_2 (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 reduced per minute per mg protein using an extinction coefficient of $6.22 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$.

Determination of GSH

GSH levels were measured using the DTNB-GSSG Reductase Recycling Method of Anderson et al. [32]. Part of the livers were homogenized in 5% sulfosalicylic acid (5 vol/g tissue), centrifuged for 5 minutes at 2500 rpm, and the supernatant was collected. All reagents were prepared using a stock buffer containing 143mM dibasic sodium phosphate and 6.3mM Na_4EDTA . Daily buffer (700 μ L), containing 0.248 mg NADPH/ml of stock buffer, 100 μ L of 6mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 175 μ L distilled water and 25 μ L of supernatant were combined in a test tube and incubated at 30°C for 15 minutes. Then, 10 μ L of glutathione reductase (266 units in stock buffer) was added to each tube to start the reaction. The change in absorbance was measured for 3 minutes at 412 nm, using a Genesys™ 20 spectrophotometer (Thermo Scientific, Madison, WI). Standard curve was prepared with reduced glutathione following the above procedure but replacing 25 μ L of supernatant with a standard solution containing 1–4 nmol reduced glutathione.

Protein Determination

The amount of protein present in each sample was measured using the method of Lowry et al. [33], using BSA as a standard, and data for various biomarkers, except for glutathione level that was determined as nmol/g tissue were expressed per mg protein.

Statistical Analysis

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

RESULTS

The production of SA, LP and DNA SSBs in the livers of mice exposed to either DCA or TCA and fed either a Std or a Low-E diet are shown in figures 1, 2 and 3, respectively. Exposure to DCA or TCA resulted in significant increases in all three biomarkers in the livers of mice in either diet group, when compared to their corresponding controls. However, the observed increases in those biomarkers in the Std diet groups were significantly greater than those of the Low-E diet groups.

The DCA and TCA treatment impacts on the hepatic SOD, CAT and GSH-Px activities of mice fed either a Low-E or Std diet are demonstrated in figure 4 A, B and C, respectively. Figure 4 A shows that the control animals in the Low-E diet group expressed significantly higher SOD activity than the control in the Std group. When compared to the corresponding control of each diet, SOD activity was suppressed in the hepatic tissues of mice exposed to DCA in either diet group, with significantly greater suppression observed in the livers of the Std diet group as compared to the Low-E diet group. TCA on the other hand resulted in increases in hepatic SOD activity of both groups when compared with their corresponding controls, with a significantly greater increase observed in the Low-E diet group, as compared with the corresponding treatment of the Std diet group. Figure 4B, while demonstrates no significant effects induced by DCA on CAT activity in any of the diet

groups, it indicates significant changes in the enzyme activity in response to TCA the two diet groups, as compared with the corresponding diet controls. The effects on GSH-Px activity in the livers of mice exposed to DCA or TCA are demonstrated in figure 4C which indicates the expression of higher enzyme activity by the livers of the Low-E diet control, as compared with the Std diet control. While figure 4C demonstrates significant suppressions in the hepatic GSH-Px activity in response to DCA and TCA in the Std diet groups, it indicates significant increases in the enzyme activities in response to the compounds' treatment in the Low-E diet groups, as compared with the corresponding diet controls.

The effects of DCA or TCA treatment of mice fed either a Low-E or Std diet on total glutathione (GSH) levels are shown in figure 5. When compared to the corresponding control in each diet group, DCA resulted in a significant suppression and significant increase in hepatic GSH activity of the Std diet and the Low-E diet group, respectively. TCA treatment on the other hand while did not result in a significant change in hepatic GSH level in the Std diet group, it did result in a significant increase in this antioxidant molecule in the Low-E diet group when compared with the corresponding controls.

Table 1 shows the final body weight (BW) on the day of sacrifice, liver weight (L), and liver: body weight (L/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 L and L/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.

DISCUSSION

SA is a ROS that is known to cause damage to different cellular structures, and the decreases in LP and DNA damage associated with decreases in DCA- and TCA-induced SA production in the Low-E diet group, compared to the Std diet group may confirm that.

Vitamin E is a well known antioxidant, and it was therefore expected that a Low-E diet consumption would increase OS induction by the compounds. However, the results contradicted that expectation. Superoxide dismutase (SOD) is an antioxidant enzyme that converts SA to H_2O_2 and is acting in concert with GSH-Px and CAT that detoxify H_2O_2 [34–36]. DCA induced less significant SOD suppression, while TCA produced greater SOD stimulation in the Low-E diet groups, as compared with the Std diet groups. This, together with DCA- and TCA-induced increases in GSH-Px activities in the Low-E diet groups, as opposed to the compounds-induced enzyme suppression the Std diet group, should account for the observed lower SA levels and its associated LP and DNA damage levels in the low-E diet groups. The increases in SOD and GSH-Px in the Low-E diet control, compared to the Std diet control, can be viewed as adaptation mechanisms to the loss of protection that is otherwise provided by vitamin E supplementation; a conclusion that is supported by the studies of Cabo et al. [37], and can be also confirmed by the similar SA levels in the controls of the two diet groups.

GSH is an important antioxidant molecule, and the observed compounds-induced increases in GSH levels in the low-E diet groups is in line with the studies of Tchanchou et al. [38] demonstrating significant increases in GSH and its associated GSH synthase activity in response to deprivation of folate and vitamin E in mice.

Besides the increases in SOD, GSH-Px and GSH, increases in CAT activity in response to TCA but not DCA in the Low-E diet group suggests a better protection against TCA than DCA effects in that group, and can be further confirmed by the greater reduction of TCA- than DCA-induced DNA damage in that same group.

Taken together, the observed increases in GSH, and GSH-Px and SOD activities and the decreases in LP and DNA in response to the compounds in mice fed Low-E diet suggests a production of protective effects in response to that diet against the compounds-induced OS, i.e., vitamin E supplementation to the diet while essential to maintain various body and cellular functions, it can compromise the effectiveness of other natural antioxidants. Furthermore, the expression of significant increases in the L and L/BW by the compounds in the Std diet groups when compared to the control of the Low-E diet group may indicate a possible increase in the compounds risk to induce hepatotoxicity when fed a Std diet. However, this suggestion should not be taken as a recommendation for non supplementation of the vitamin to the diet, as partial protection against the compounds-induced OS/ hepatotoxicity must be weighed against the possible loss of other essential effects of the vitamin on different body and cellular functions.

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Figure 1. 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 2.

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 different ($p < 0.05$).

Figure 3. 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 4. SOD activity (units/mg protein) (A), CAT activity (Units/ mg protein) (B), and GSH-Px activity (nmoles NADPH oxidized/min/ mg protein) (C) 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 5. Total GSH (nmol/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

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.

	BW		L (g)		L/BW	
	Std	Low-E	Std	Low-E	Std	Low-E
Control	34.0 ± 2.1	33.6 ± 1.8	1.8 ± 0.3	1.5 ± 0.2 ^{a,b}	0.052 ± 0.006	0.044 ± 0.006 ^{a,b}
DCA	34.4 ± 2.3	34.0 ± 2.3	1.9 ± 0.2 ^{a,c}	1.6 ± 0.2 ^c	0.056 ± 0.003 ^{a,c}	0.046 ± 0.005 ^c
TCA	35.3 ± 0.99	34.9 ± 2.5	2.0 ± 0.2 ^b	1.7 ± 0.2	0.057 ± 0.003 ^b	0.050 ± 0.006