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The Induction of Tumor Necrosis Factor-alpha , Supeoxide Anion, Myeloperoxidase, and Superoxide Dismutase in the Peritoneal Lavage Cells of Mice after Prolonged Exposure to Dichloroacetate and Trichloroacetate

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Abstract

The induction of phagocytic activation in response to prolonged treatment with different doses of dichloroacetate (DCA) and trichloroacetate (TCA) has been investigated in mice. Groups of B6C3F1 male mice were administered 7.7, 77, 154 and 410 mg of DCA or TCA/ kg/day , post orally, for 4- and 13-weeks. Peritoneal lavage cells (PLCs) were isolated and assayed for the different biomarkers of phagocytic activation, including superoxide anion (SA), tumor necrosis factor-alpha (TNF- α), and myeloperoxidase (MPO). In addition, the role of superoxide dismutase (SOD) in the SA production was also assessed. DCA and TCA produced significant and dose-dependent increases in SA and TNF- α production and in MPO activity but the increases in response to the high doses of the compounds (> 77 mg/kg/day) in the 13-week treatment period were less significant than those produced in the 4-week treatment period. Also, dose-dependent increases in SOD activity were observed in both periods of treatments. In general, the results demonstrate significant induction of the biomarkers of phagocytic activation by doses of DCA and TCA that were previously shown to be non carcinogenic, with significantly greater increases observed at the earlier period of exposure, as compared with later period. These findings may argue against the contribution of those mechanisms to the hepatotoxicity/hepatocarcinogenicity of the compounds and suggest them to be early adaptive/ protective mechanisms against their long term effects.

Keywords

Dichloroacetate; Trichloroacetate; Peritoneal lavage cells; Subacute; Subchronic; Superoxide anion; Tumor necrosis factor-alpha; Myeloperoxidase

Introduction

Dichloroacetate (DCA) and trichloroacetate (TCA) are among the by-products formed in the water during the process of water chlorination (1–3). They are also known to be formed as metabolites of trichloroethylene, a widely used industrial solvent and water contaminant (4–7). The concentrations of TCA and DCA in the municipal water supplies are in the range of 30–160 $\mu\text{g/l}$ (2,8), and those together with possible exposure to the compounds from other environmental sources, suggest risk potential from long term exposure.

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Hepatotoxicity and hepatocarcinogenicity have been demonstrated as the most prominent effects of DCA and TCA in rodents, after long term exposure (9–12). Biomarkers of oxidative stress, such as production of lipid peroxidation, superoxide anion (SA) production and DNA damage were found to be induced in the hepatic tissues of mice exposed to single high doses of the compounds and have been suggested as possible mechanisms for the compounds'-induced hepatotoxicity and hepatocarcinogenicity (13–18).

Activated phagocytic cells are known to be associated with the production of ROS, such as superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (19–23), the release of the enzyme myeloperoxidase (MPO) (24–26), and the production of tumor necrosis factor alpha (TNF- α) (27–29). While those products are known to play a major role in the host defense against bacterial infections and tumors, they are also known to mediate tissue damage under certain circumstances, especially during the processes of inflammation and ischemic injury (25,30–31).

DCA and TCA were found to induce dose- and time-dependent production of SA in the J774.A1 macrophage cells (32–33). *In vitro* studies on the effects of the compounds in the J774A.1 macrophage cells have also assessed the protective effects of antioxidant enzymes and TNF antibodies against the compounds'-induced SA production (33–34). Recent studies have demonstrated the production of phagocytic activation in the peritoneal lavage cells (PLCs) of B6C3F1 male mice, several hours after exposure to single high doses of DCA and TCA (18).

Based on the above mentioned *in vivo* acute studies, as well as *in vitro* studies indicating the induction of phagocytic activation by DCA and TCA, and in an attempt to correlate the induction of this mechanism with the long term hepatotoxicity and carcinogenicity of the compounds, the study was designed to determine different biomarkers of phagocytic activation in the PLCs of B6C3F1 mice after 4 weeks (subacute) and 13 weeks (subchronic) exposure to doses of DCA and TCA that were previously found to range between the non hepatotoxic/hepatocarcinogenic and those producing maximal hepatotoxicity/hepatocarcinogenicity. The results of the study were presented in part, in the Society of Toxicology (SOT) meeting (35).

Materials and Methods

Chemicals

All of the chemicals, including sodium dichloroacetate (DCA) and sodium trichloroacetate (TCA) were purchased from Sigma Chemical Company (St. Louis, MO), and were of the highest grades available.

Animals and treatments

B6C3F1 male mice were used for this study, based on previous studies that found the animals to be sensitive to the acute, as well as the long term effects of DCA and TCA (9–18). The animals were about 6 weeks of age and weighing approximately 20 g when they received the first doses of the compounds. They were allowed to acclimate for 3 days prior to the experimental use, caged at 21° C with a 12 hr light/dark cycle, maintained on a standard laboratory chow from Harlan Teklad (Madison, Wisconsin), and allowed a free access to food and water. DCA and TCA were dissolved in distilled water, and pHs of the solutions were adjusted to 7.0 by sodium hydroxide solution. Groups of mice (7 animals/ group) were treated post orally, with daily doses of 7.7, 77, 154, and 410 mg/kg body weight of DCA or TCA, for 4 weeks (subacute treatment) and 13 weeks (subchronic treatment). These doses were based on previous studies that investigated the abilities of the compounds to induce hepatotoxic and hepatocarcinogenic effects in B6C3F1 male mice provided with drinking water containing the compounds at concentrations ranging between 0.05–5 g/l of the compounds for 60–75 weeks

(9,11–12). DeAngelo et al. (11) have calculated the time-weighted mean daily doses that correspond to 0.05, 0.5, 3.5 and 5 g/l of DCA in the drinking water and found them to be equivalent to 7.6, 77, 410 and 486 mg/kg/day, respectively, and that concentrations equivalent to 7.6, 77, and 410 mg/kg/day are the doses that correspond to the non carcinogenic dose, the threshold carcinogenic dose and the dose that results in 100% tumor prevalence, respectively. Also, the dose-response of those studies has demonstrated a steep rise to a maximum tumor incidence at 2 g/l DCA, immediately after the threshold concentration of 0.5 g/l (11). Similarly, Bull et al (12) have indicated production of hepatoproliferative lesions (HPL) in male B6C3F1 mice exposed to drinking water containing 1 or 2 g/l DCA or TCA and found that the induction of HPL by TCA was linear with the dose, while that of DCA increased sharply with the increase from 1 to 2 g/l. Furthermore, Herren Freund et al. (9) have demonstrated DCA and TCA as complete carcinogens in B6C3F1 where hepatocellular carcinomas were produced in 81 or 32% of the animals exposed to drinking water containing 5 g/l of DCA or TCA, respectively. The studies also demonstrated increases in the incidence of animals with adenomas in response to those treatments. Control animals received distilled water at a rate of 5.0 ml/kg body weight/day, after adjusting its pH to 7.0 with sodium hydroxide solution. Animals were euthanized at the end of the treatment periods, using carbon dioxide anesthesia followed by cervical dislocation.

Collection of the peritoneal lavage cells (PLCs)

The PLCs were collected by injecting 3 ml of a buffer containing 140 mM NaCl, 5mM KCl, 10 mM glucose, 20 mM HEPES and 2 mM CaCl₂, (pH 7.3), into the peritoneal cavities of the animal, immediately after sacrifice. The peritoneal cavities were massaged to release the cells, and fluids were withdrawn from the peritoneal cavities by a syringe. The PLCs suspensions were centrifuged at $1700 \times g$ for 10 minutes and cell pellets were re-suspended in 2 ml of DMEM containing methionine and supplemented with glutamine, penicillin-streptomycin, Hepes buffer, MEM-NEAA, sodium pyruvate solution and fetal bovine serum (FBS). Aliquots of 200 μ l of cellular suspensions were plated on 96-well plate, and were incubated and used for the determination of tumor necrosis factor-alpha (TNF- α) production as described below. The rest of the cellular suspensions were used for the determination of SA production and myeloperoxidase (MPO) and superoxide dismutase (SOD) activities.

Determination of TNF-alpha

Plates containing cellular suspensions were incubated for 24 h at 37° C in a humidified atmosphere containing 5% CO₂. Media were then collected and assayed for TNF- α production using the Quantikine mouse TNF- α Immunoassay, which is a 4.5 h solid phase ELISA (R & D Systems, Minneapolis, MN). The assay was conducted following the procedure indicated in the kit and plates were read in a microplate reader set to 450 nm. TNF- α levels were determined according to a standard curve prepared with serial dilutions of recombinant murine TNF- α standard provided with the kit.

Determination of superoxide anion (SA)

SA production was determined in the PLCs, using the cytochrome *c* reduction assay (21), with modifications. In brief, the 2 ml reaction mixture contained 50 μ l of PLCs suspension and 0.05 mM cytochrome *c* oxidase in phosphate buffered saline (PBS), pH 7.2. Reaction mixtures were incubated for 15 minutes at 37° C, after which reactions were terminated by placing the tubes on ice. The mixtures were centrifuged at 3000 rpm for 15 minutes and absorbances of the supernatant fractions were measured at 550 nm, using Spectronic 20 spectrophotometer. 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 MPO activity

MPO activity was measured according to the method of Bradley et al. (26). Aliquots of 0.5 ml cellular suspensions were freeze-thawed 3 times and were then centrifuged at 4,000 g for 15 min and the resultant supernatant was assayed. The assay mixture contained 0.1 ml of supernatant mixed with 2.9 ml of 50 mM Phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in the absorbance was measured at 460 nm for 5 minutes, and absorbances were converted into units of MPO activity, where one unit is defined as that degrading one micromole of hydrogen peroxide per minute at 25° C. A molar extinction coefficient of 11300 for the oxidized o-dianisidine was used.

Determination of SOD activity

Aliquots of 0.5 ml cellular suspensions were centrifuged at 3000 g for 10 min and cell pellets were re-suspended in 2 ml sucrose buffer containing 0.32 M sucrose, 1 mM EDTA, and 10 mM Tris-HCL. Cellular suspensions were freeze-thawed 3 times and were then centrifuged at 4,000 g for 15 min and the resultant supernatant was assayed for SOD activity according to the method of Marklund and Marklund (36). In brief, an aliquot of the supernatant (200 µl) was mixed with 750 µl Tris-cacodylic buffer, containing 50 mM Tris-HCL, 50 mM cacodylic acid and 1 mM EDTA, pH 8.2, followed by the addition of 250 µl of 2 mM pyrogallol. Absorbances of the mixtures were recorded at 420 nm immediately and then every 30 sec for 3 min, using a Spectronic 20 spectrophotometer. Changes in the rate of absorbances were calculated and converted into units of SOD activity, where one unit is equivalent to the quantity of SOD that is needed to produce 50% inhibition of pyrogallol autooxidation (36).

Determination of protein

The amounts of protein in the cellular suspensions were determined according to the method of Lowry (37), using bovine serum albumin as a standard, and data for SA, SOD, MPO and TNF- α , were expressed as nmoles/min, units, units and pg, respectively, /mg protein.

Statistical methods

Data were analyzed using Microsoft Excel® data analysis tool package. Data are expressed as means of 7 samples (animals)/dose/compound/time point $\pm$ S.D. A one-way Analysis of Variance (ANOVA) was used to determine the statistical differences between groups, with Scheffe's S method used as a post hoc test. A significance level of $p < 0.05$ was employed.

Results

The effects of DCA and TCA on SA production in the PLCs are demonstrated in figures 1A and B, respectively. Dose-dependent increases in SA production were observed with DCA and TCA doses higher than 7.7 mg/kg/day in the 4-week treatment period. While both compounds produced significant increases in this biomarker at doses ranging between 7.7–154 mg/kg/day in the 13-week treatment period, the level of the biomarker declined at a dose of 154 mg/kg of either compound, as compared with the immediately lower dose and reached the corresponding control level at a dose of 410 mg DCA or TCA/kg/day.

Figures 2A and B present the effects of DCA and TCA, respectively, on SOD activity. DCA and TCA resulted in significant and dose-dependent increases in SOD activity with doses ranging between 7.7–410 mg/kg/day of the 4-week treatment period, and with doses ranging between 7.7–410 mg/kg/day at the 13-week treatment period. The figures also indicate significantly greater increases in SOD activity in response to DCA and TCA at the 13-week period, as compared with the 4-week treatment period.

Figures 3A and B demonstrate the effects of DCA and TCA, respectively, on MPO activity. DCA and TCA resulted in significant increases in MPO activity when administered at doses ranging between 7.7–410 mg/kg/day in the 4-weeks treatment period, as compared with the corresponding control. While significant increases in MPO activity were also observed in response to DCA and TCA doses ranging between 7.7–154 mg/kg/day administered for 13 weeks, no significant changes in MPO activity were observed in response to 410 mg/kg/day of either compound when compared with the corresponding control at that period of treatment. Figure 3B also shows that the increases in response to 7.7–154 mg of TCA/kg/day at the 13 weeks period were significantly less than those produced in response to the same doses at the 4 weeks period.

The effects of DCA and TCA on TNF- α production are demonstrated in figures 4A and B, respectively. Dose-dependent increases in TNF- α production were observed with DCA doses ranging from 7.7–410 and 7.7–154 mg/kg/day at the 4- and 13-week exposure periods, respectively (figure 4A). While TCA administration at doses ranging between 7.7–410 mg/kg/day resulted in dose-dependent increases in TNF- α production in the 4-weeks treatment period, significant production of this cytokine was only observed in response to 154 mg TCA/kg/day in the 13-weeks treatment period when compared with the corresponding control (figure 4B).

Table 1 shows the results of comparisons between the effects of DCA and TCA on the different biomarkers. Data of the 7 samples for the effect of DCA on each biomarker, in response to each dose and at each time point were pooled and compared with the corresponding pooled data for the same biomarker, dose and time point for TCA, using one-way ANOVA. The p-values were calculated and a significance level of $p < 0.05$ was employed. In general, DCA produced significantly greater effects than those of TCA on SA production at both time points, and on SOD, MPO and TNF- α , in response to a certain dose level at the 13-week treatment period. TCA on the other hand produced significantly greater effects than those of DCA on MPO activity and TNF- α production, at the 4-week treatment period.

Discussion

The results of this study indicate the induction of phagocytic activation in response to subacute and subchronic administration of DCA and TCA to mice, as assessed by three important biomarkers of phagocytic activation that included SA and TNF- α production and MPO activity. Phagocytic activation is known as a defense mechanism against bacterial infections, but it can be associated with the destruction of normal or neoplastic cells (30,22–23,27,38–39). Since hepatotoxicity and hepatocarcinogenicity are the most prominent injuries produced by DCA and TCA in B6C3F1 male mice after long term exposure (9,11–12), it is most likely that phagocytic activation occurs in response to those injuries. DCA and TCA induced significant SA production in response to subacute and subchronic treatment with 7.7–410 mg/kg/day and 7.7–154 mg/kg/day, respectively. Superoxide dismutase (SOD) is not a biomarker of phagocytic activation, but is an antioxidant enzyme responsible for SA dismutation and its conversion to H_2O_2 (40), and was determined for this study to better understand the profile of SA production in response to the compounds. TCA and DCA administration to the mice resulted in dose-dependent increases in SOD activity in the two tested treatment periods. This may indicate possible up-regulation of the enzyme to protect against SA overproduction and may also suggest possible role for H_2O_2 in the observed effects. Significantly greater increases in SOD activity were observed in response to different doses of the compounds in the 13-week, as compared with the 4-week treatment period which may account for the significantly less SA production, especially in response to subchronic treatment with 154–410 mg/kg/day of DCA or TCA, when compared with the corresponding subacute treatment. Despite the significantly greater increase in SOD activity in response to some of the DCA doses when compared with TCA during the two periods of treatment, DCA resulted in significantly greater

increases in SA production at both periods, as compared with TCA. These findings are inline with those of previous studies testing the effects of single acute doses of DCA and TCA on the production of SA by the PLCs (16), and SA and other biomarkers of oxidative stress in the hepatic tissues of mice (16,18), as well as of studies comparing the effects of various concentrations of the compounds on SA production by the macrophage cells in culture (32–33).

MPO has been shown to act as immunoregulator, both *in vivo* and *in vitro*, inducing the release of ROS, TNF- α and interferon alpha/beta (41–42), and the results of the study clearly demonstrate parallel increases in MPO activity and SA and TNF- α production, in response to different doses of the compounds, at both periods of treatment. Reaction of MPO with H₂O₂ in the presence of physiological concentrations of Cl⁻ results in the formation of the powerful oxidant hypochlorous acid/hypochlorite (HOCl/OCl⁻) (24,26,43–44), and components of the peroxidase-H₂O₂-halide system, including MPO have been shown to contribute to the killing of certain mammalian tumor cells (45). The results of the study indicate less significant increases in MPO activity in response to DCA and TCA doses ranging between 154–410 and 7.7–410 mg/kg/day, respectively, in the 13-week treatment period when compared with the corresponding doses at the 4-week exposure period. The decline in this biomarker in the later period of treatment as compared with the earlier period may suggest an initial and early protective role for MPO against DCA- and TCA-induced injuries.

TCA-induced increases in MPO activity and TNF- α were significantly greater than those induced by DCA in the 4-week treatment period. On the other hand, DCA-induced increases in those biomarkers were significantly greater than those induced by TCA in the 13-week treatment period. Studies involved monitoring H₂O₂ uptake by MPO have shown that when the concentration of this species is increased to a certain level it causes inhibition of MPO activity, but Cl⁻ decreases H₂O₂ effects on the enzyme (44). Also, Kettle and Winterbourn (43) have proposed the contribution of SA generation by the neutrophils to the prevention of H₂O₂ and other one-electron donors from inhibiting MPO. Furthermore, TCA undergoes a one-electron reduction and hemolytic cleavage during the process of metabolism, giving rise to DCA and Cl⁻, and DCA can also generate Cl⁻ through a similar process, giving rise to monochloroacetic acid (16). Although this can lead to the conclusion that the changes seen in MPO activity were associated with the net effect of variable concentrations of Cl⁻ generated during the process of metabolism of the compounds, SA produced in response to the compounds and the concentration of H₂O₂ formed in response to SA dismutation by SOD, it is difficult to assess the exact contribution of each species to those changes at this point.

Lefkowitz et al, (42) have demonstrated the role of MPO in the *in vitro* release of TNF by the mouse peritoneal macrophages, and also in the *in vivo* release of the cytokine in the sera of mice, and they suggested radical production by MPO or binding of MPO to the mannosyl-fucosyl receptor as possible mechanisms for the TNF release. This finding supports the results of this study showing similarities in the profiles of MPO and TNF- α in response to different doses of DCA and TCA, as well as the profile for the differences between the two compounds' effects on the two biomarkers, in the two treatment periods.

Wang and Lin (46) have described TNF as a “double-dealer”, where it can act as an endogenous tumor promoter or a cancer killer, depending on the cross talk between the two signals it initiates: the nuclear factor- κ B, which is a major cell survival signal that is anti-apoptotic, and c-Jun N-terminal kinase that is considered as a cell death signal. Less significant increases in TNF- α production were observed with DCA and TCA doses ranging between 154–410 and 7.7–410 mg/kg/day, respectively, in the 13-week exposure period when compared with the corresponding doses of the compounds in the 4-week period. Those observations are inline with those of MPO and may also suggest an initial contribution of the cytokine release to the

protection against injury, rather than to toxicity production by the compounds. i.e., have those biomarkers contributed to the toxicity rather than to the protection against injury, significantly greater increases would have been observed with the higher doses and the longer period of treatment.

Studies on the tumorigenesis by DCA and TCA have suggested two different mechanisms for the compounds, with DCA effect depends on the stimulation of cell division secondary to hepatotoxic damage, and TCA effect is associated with the production of free radicals and the induction of DNA repair synthesis (12,47). Studies on the mechanism of tumorigenicity by the compounds(12) have also indicated production of hepatoproliferative lesions (HPL) in the livers of mice after 52 weeks of treatment with the compounds, and that suspension of the treatment at 37 week of treatment would result in similar number of HPL lesions in response to DCA treatment for 52 weeks, but of significantly lower number of lesions in response to TCA treatment for 52 weeks, indicating possible reversibility of the damage. In another study, it has been indicated that lower doses of DCA may also involve damage to DNA (47), and that will also indicate possible repair and reversibility in response to those doses. These may all support our suggestion that lower doses and earlier periods of treatment are associated with some protective/ adaptive mechanisms, and phagocytic activation could also contribute to those mechanisms.

Previous studies in our lab have demonstrated the induction of phagocytic activation and the production of oxidative stress in the hepatic tissues of B6C3F1 mice, several hours after the administration of single high doses on DCA and TCA and suggested the contribution of phagocytic activation to the induction of oxidative stress in the hepatic tissues (18). That suggestion contradicts the conclusion made in this study about the possible protective role of phagocytic activation against DCA- and TCA-induced cellular injuries. The reasons for the those contradictions include, the current detailed studies that involved studying various markers of phagocytic activation, compared to SA production as the only marker used to assess the role of that mechanism in the previous studies (18). Also, no assessment was made for the role of SOD in SA production in the previous studies. Furthermore, the current studies have determined the effects of a wide range of doses and after two prolonged periods of treatment, compared with the effects of brief exposure to single doses of the compounds that were determined in the previous one. The results of this study also differ from those of *in vitro* studies showing an association between SA production and cellular death induced by DCA and TCA on macrophage cell culture. These differences are clearly related to the toxicokinetic of the compounds in an *in vivo*, as compared with an *in vitro* system (32–34)

In conclusion, and contrary to the expected dose- and time-dependent increases in the biomarkers of phagocytic activation that had been primarily proposed to contribute to the hepatotoxicity/hepatocarcinogenicity of the compounds, the results generally indicated more increases in those biomarkers in the 4-weeks treatment period than the 13-weeks period, and also in response to the lower doses than the carcinogenic ones during that period. These observations may indicate that the compounds'-induced phagocytic activation does not contribute to their hepatotoxicity/hepatocarcinogenicity, but instead is suggested as an initial and early mechanism induced to adapt to, and/ or protect against the compounds'-induced primary injuries, but was then overwhelmed and started to decline in response to the higher doses and/ or the longer periods of treatment . Studies on the compounds' induced hepatotoxicity and oxidative stress in the livers of mice treated with similar doses and periods of treatments are underway in our laboratory to assess that.

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Figure 1.

A and B: SA production, determined as cytochrome c reduced/min/mg protein in the PLCs of the (A) DCA-treated mice, and (B) TCA-treated mice, 4 weeks (4-W) and 13 weeks (13-W) after exposure. Columns that do not share an identical superscript are significantly different ($p < 0.05$)

Figure 2.

A and B: SOD activity determined in the PLCs of the (A) DCA-treated mice, and (B) TCA-treated mice, 4 weeks (4-W) and 13 weeks (13-W) after exposure. Columns that do not share an identical superscript are significantly different ($p < 0.05$)

Figure 3.

A and B: Myeloperoxidase activity determined in the PLCs of the (A) DCA-treated mice, and (B) TCA-treated mice, 4 weeks (4-W) and 13 weeks (13-W) after exposure. Columns that do not share an identical superscript are significantly different ($p < 0.05$)

Figure 4.
A and B: Amount of TNF- α released by the PLCs of mice in response to (A) DCA and (B) TCA treatment for 4 weeks (4-W) and 13 weeks (13-W). Columns that do not share an identical superscript are significantly different ($p < 0.05$)

Table 1

Comparisons between the responses to DCA and TCA at each of the tested doses at the 4-weeks (4-W) and 13-weeks (13-W) treatment periods. Numbers indicate the calculated p-values for the differences between the effects of the two compounds on each biomarker, in response to each dose and each time of treatment, where p-values < 0.05 indicate a statistically significant difference.

		mg/kg/day			
		7.7	77	154	410
<u>Biomarker</u>					
SA	(4-W)	0.9770	0.0004 *	$1.4 \times 10^{-5} *$	$8 \times 10^{-6} *$
	(13-W)	0.0002 *	0.0005 *	$2.0 \times 10^{-6} *$	0.0320 *
SOD	(4-W)	0.5330	0.1900	0.7200	0.0002 *
	(13-W)	0.9500	0.0015 *	0.0410 *	0.5700
MPO	(4-W)	0.0250 **	0.0020 **	0.0036 **	0.1800
	(13-W)	$5.8 \times 10^{-5} *$	0.2500	$1.1 \times 10^{-6} *$	0.2100
TNF	(4-W)	0.7200	0.0105 *	0.0102 *	0.0007 *
	(13-W)	0.0002 *	$4 \times 10^{-12} *$	$8.1 \times 10^{-11} *$	0.3000

* DCA effect is greater than TCA effect
** TCA effect is greater than DCA effect.