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Dichloroacetate- and trichloroacetate-induced cellular death and oxidative stress in AML-12 hepatocytes

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A Thesis

Entitled

Dichloroacetate- and Trichloroacetate-Induced Cellular Death and Oxidative Stress in AML-12 Hepatocytes

by

Christopher Mettling

Submitted to the Graduate Faculty as partial fulfillment of the requirements

for the Master of Science Degree in Pharmaceutical Science

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An Abstract of

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The water chlorination process results in the production of different haloacetates that have been found to be toxic. Previous *in vivo* studies in animals have reported several effects, including hepatotoxicity, carcinogenicity and induction of oxidative stress by two important haloacetates produced during this process, dichloroacetate (DCA) and trichloroacetate (TCA). This study focused on AML-12 hepatocyte cytotoxicity and induction of oxidative stress resulting from exposure to DCA and TCA, in an effort to establish an *in vitro* system to test the effects of these and other haloacetates, as well as mixtures of haloacetates.

Cell cultures were exposed separately to varying DCA and TCA concentrations and incubated for 24, 48, and 72 hours. Cellular toxicity was assessed by determining cellular viability, and oxidative stress was assessed by three biomarkers, including superoxide anion (SA) and lipid peroxidation (LP) production, and superoxide dismutase (SOD) activity. The results of the study demonstrate concentration- and time-dependent effects on the production of cellular death and various biomarkers of oxidative stress by DCA and TCA, similar to what is observed *in vivo*, after long term exposure. The results also demonstrate that the effective concentrations of the compounds are exactly a factor of ten times greater than the doses required for the

production of various levels of hepatotoxic and hepatocarcinogenic, as well as biomarkers of oxidative stress in animals, and suggest the cells to be an appropriate model for testing the effects of various individual haloacetates, and mixtures of haloacetates.

Acknowledgements

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For E. and R. Bader.

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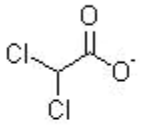
Introduction

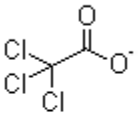
I. Water Chlorination

Chlorine, as a chemical disinfectant of drinking water, has reduced and prevented water borne diseases since its introduction in the 20th century. It has become standard practice in America, despite a number of studies highlighting its harms (Uden and Miller, 1983). Chlorinated disinfectant by-products, known as DBPs, are subject to debate following their role in adverse effects following animal testing. Chlorine reacts quickly with many natural organic compounds due to high reactivity, producing chloroform and chlorinated aliphatic acids (Peters, 1991). Nine halogenated acetic acids (HAAs) are formed in this process, though only five species are regulated; including dichloroacetate (DCA) and trichloroacetate (TCA) (Liang and Singer, 2003).

II. Halogenated Acetic Acids (dichloroacetate and trichloroacetate)



Dichloroacetate
(DCA)



Trichloroacetate
(TCA)

The formation of HAAs depends on a number of factors, including pH, reaction time, and temperature (Liang and Singer, 2003; Zhuo et al., 2001). A shift in pH from 6 to 8 results in reduced TCA level, but has no impact on DCA. As contact time with chlorine increases, more and more precursors are decomposed, and more DBPs can accumulate. Temperature can greatly

affect production, with lower producing significantly less DBPs than higher temperatures (Zhuo et al., 2001).

DCA and TCA are known human metabolites of the industrial solvent trichloroethylene (Prout et al., 1985; Hathway, 1980). DCA and TCA sources also include agricultural use as herbicides and industrial, as laboratory reagents. Concentrations found in municipal water supplies range from 30 to 160 µg/l (Jolley, 1984; Miller and Uden, 1983).

DCA is also used as a therapeutic drug and preparations created with sodium gluconate and glycine to produce a DCA moiety labeled as vitamin B_{15} (Stacpoole, 1989). In diabetes mellitus, the compound was sought for its ability to inhibit gluconeogenesis and reduce hyperglycemia, while inhibiting lipogenesis and cholesterolgenesis. Currently, it is used in the treatment of lactic acidosis.

A. Dichloroacetate and Trichloroacetate Toxicity and Toxicokinetics

Long term carcinogenicity and toxicity studies on DCA and TCA revealed that both compounds are hepatocarcinogenic and hepatotoxic in B6C3F1 mice (Daniel et al., 1992; DeAngelo et al., 1991; Herren-Freund et al., 1987; Pereira, 1996). Neither DCA nor TCA is mutagenic in the Ames test (Stauber and Bull, 1997), although *in vivo* they are capable of producing single-strand breaks in DNA of hepatic tissue (Bull et al., 1990). Both DCA and TCA have an early stimulatory effect on cell replication in normal hepatocytes, and with chronic treatment, cell replication becomes significantly reduced (Sanchez and Bull, 1990; Stauber and Bull, 1997). At a given dose, DCA results in more frequent benign tumors, whereas a higher fraction of tumors produced by TCA are malignant (Bull et al., 2002; Bull, 2000).

Initial toxicological testing revealed that doses up to 250 mg/kg/day DCA in rodents were nontoxic (Neal, 1981) and acute oral LD-50 in mice has been reported as 5520 mg/kg (Woodard

et al., 1941). More recent studies (Bull et al., 1990; DeAngelo et al., 1991; Daniel et al., 1992; Leavitt, 1997; Pereira, 1996) have shown significant hepatic tumor formation can occur within the 250 mg/kg/day dose. Daniel et al. (1992) showed that 78% of mice treated with 88 mg/kg/day of DCA for 104 weeks developed tumor growth. Herren-Freund et al. (1987) observed that after 61 weeks of exposure to 486 mg/kg/day DCA, hepatocellular carcinomas were found in 81% of animals. DeAngelo et al. (1991) established a range of DCA doses in mice concerning hepatocarcinogenicity, varying from 7.6 to 486 mg/kg day^{-1} for 60-75 weeks, with 77 mg/kg/day identified as the threshold carcinogenic dose. Chronic dosing of 410 mg/kg /day for 60 weeks showed a 100% tumor prevalence rate, which was not significantly different from the highest tested dose (486 mg/kg/day).

Animals treated with DCA doses of 300 mg/kg/day for 52 weeks exhibited focal necrosis and showed significant increases in the size and weight of the liver, associated with massive accumulation of glycogen. Cellular proliferation was also found to surround the necrotic areas, suggesting that necrosis produced by DCA, along with inherent hepatomegaly and cytomegaly, may be a factor in tumorigenesis (Larson and Bull, 1992; Bull et al., 1990).

When DCA was given orally to rodents, less than 2% of any dose was recovered in urine as the parent compound. DCA metabolism occurs in the cytosol of hepatic cells and metabolized to carbon dioxide, glycine, glycolate, and oxalate, utilizing glutathione (Tong et al., 1998).

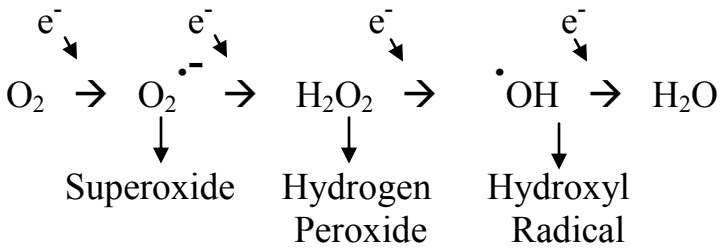
TCA was also found to be hepatotoxic and hepatocarcinogenic in mice after long term exposure (DeAngelo, 1989; Herren-Freund et al., 1987; Pereira, 1996). Herren-Freund found that 61 weeks of exposure to 486 mg/kg/day TCA stimulated hepatocellular carcinomas in 32% of animals. Bull et al. (1990) showed that 20% of mice treated with 150-300 mg/kg/day for 37-52 weeks developed carcinogenic tumors. TCA tumorigenicity was found to be dose-dependent

with no abnormal cell division (Larson and Bull, 1992) and infrequently occurring necrotic lesions (Bull et al. 1990). Small increases in cell size and significantly less accumulation of glycogen occur after exposure to TCA, as compared with DCA (Bull et al., 1990). Also, TCA pathological effects in the livers of rodents are less severe than those produced by DCA (Larson and Bull, 1992). TCA was found to be metabolized to DCA and 50% of the original compound was found to be excreted in the urine as unchanged (Larson and Bull, 1992). DeAngelo et al. (1989) showed that DCA and TCA are inducers of peroxisomes in mice, with TCA found as a stronger inducer than DCA.

III. Oxidative Stress

While oxygen is essential for life, it can be toxic due to its potential to produce oxidative stress. Oxidative stress stems from the basic properties of oxygen, with two unpaired electrons as molecular oxygen, and the more reactive unpaired atomic oxygen. In both states, oxygen acts as an oxidant free radical, capable of reduction in a number of ways. When protonated or reduced forms of oxygen are created, the products are known as reactive oxygen species (ROS) (Rice-Evans et al., 1995). The body has a natural balance between oxidants and antioxidants and when this balance favors the oxidants side, it will lead to ROS overproduction and oxidative stress (Sies, 1997). ROS are potent oxidants and can attack many essential cellular components such as lipids, proteins, and nucleic acids (Fridovich, 1978; Rice-Evans et al., 1995). Exposure to free radicals and other ROS have been linked to mutagenesis, cell transformation, and cancers.

A. Superoxide Anion



Superoxide anion (SA) is the first ROS produced by the electron transport chain in mitochondria. The molecule has a half life measureable in days, which is the longest among other ROS, and is the precursor for the other more reactive species. Evidence to date illustrates that SA can react with proteins that contain transition-metal groups, resulting in loss of protein/enzyme function (Rice-Evans et al., 1995).

Superoxide dismutase (SOD) is an antioxidant enzyme responsible for SA dismutation to hydrogen peroxide (H_2O_2), which is more reactive than SA. H_2O_2 can also generate the hydroxyl radical that is considered to be the most reactive species. However, two other antioxidant enzymes work in concert with SOD, namely catalase and glutathione peroxidase, converting H_2O_2 to water (Rice-Evans et al., 1995).

B. Lipid Peroxidation

The detection and measurement of lipid peroxidation is the indication most frequently cited to support evidence of free radical reactions in toxicology. ROS can react with the lipid layer of biological membranes causing lipid peroxidation, which is characterized by loss of fluidity, lowered membrane potential, and increased permeability that can lead to cell rupture (Gutteridge, 1995). Persistent oxidation of lipid membranes results in several disease states

including cancer, rheumatoid arthritis, as well as in the degenerative processes associated with aging.

Objectives

The haloacetates are byproducts produced during the water chlorination process. Previous long term toxicity studies have found two of those haloacetates, namely dichloroacetate (DCA) and trichloroacetate (TCA) to be hepatotoxic/hepatocarcinogenic in rodents. Additional studies have found that doses required to produce those long term effects in mouse liver can also induce various levels of oxidative stress in the same tissues. These levels of oxidative stress are induced earlier than the production of long term effects effects. The studies have concluded that biomarkers of oxidative stress can be used as early indicators for the long term hepatoxicity/hepatocarcinogenicity of the compounds and that those biomarkers can be also used as better end points for the purpose of risk assessment of the haloacetates. Since there are several other haloacetates that need to be tested, and if those tests were to be done on animals, they will be challenged by ethical issues due to the need of large animals for those studies. Cost and time of animal preparation and assaying required also presents a challenge to complete those studies for every single by product, in addition to the test of mixtures of the byproducts. Such issues mandate the need for an alternative model that can be used to achieve the goal of replacing, refining and reducing (RRR) *in vivo* toxicology studies. Therefore, the AML-12 cell line, which consists of mouse hepatocytes, was tested for responsiveness to DCA and TCA effects so that the resultant *in vitro* effects could be compared with those previously determined *in vivo*.

Materials and Methods

All chemicals and reagents used for the study were purchased from Sigma Aldrich (St. Louis, MO), and were of the highest grades available.

I. Alpha Mouse Liver-12 (AML-12)

The hepatocyte cell line, AML-12, is a naked mouse cell line that possesses nontumorigenic properties. The cell line has typical hepatocyte features, including peroxisomes, gap junction, albumin, and maintains integrity after extensive passaging (Wu, 1994). It has been modified to include human transforming growth factor α (TGF-α) to stimulate initial growth (Wondergem, 2001). The cell line was originally purchased from American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown in 7 mL Dolbecco's modified Eagle - Ham medium (DMEM) (50:50), a cell culture medium supplemented with 10% FBS, 0.005 mg/mL insulin, 40 ng/mL dexamethasone, 0.005 mg/mL transferrin, and 5 ng/mL selenium per 900 mL DMEM. Cells were then incubated at 37 C° in a humidified incubator containing 5% CO_2. When cells become confluent, passage was performed. Medium was removed and 3 mL of 0.25% trypsin/EDTA was added to each flask and incubated for 10-15 minutes. Seven mL of medium was then added to neutralize the trypsin and cellular suspension was transferred from each flask to centrifuge tubes. Tubes containing cellular suspensions were then centrifuged for 10 minutes at 3000 rpm and supernatants were then removed and 10 mL of medium added to each tube containing the cellular pellets. Depending on the number of cells obtained, cellular suspensions were split among multiple flasks and additional medium was added to each flask.

The flasks were incubated at the conditions described above to be used later for cellular toxicity tests, as described below.

II. Cellular Treatments

Cells were removed from flasks and cellular suspensions were prepared as described above. Cellular suspensions containing 125,000 cells/ 1.9 mL were added to each well in 6-well plates and incubated for 12 h to allow cellular adherence and stability before they were treated. Sodium dichloroacetate (DCA) and sodium trichloroacetate (TCA) were dissolved in medium containing the above described composition and were added to different cultures at a volume of 100 µl/ well to obtain final concentrations of 4100, 1540 and 770 ppm of DCA or TCA. Control cultures were also used, where 100 µl of medium/well was added. Control and treated cultures were incubated for periods of 24, 48 and 72 h under the above described conditions. Four cultures per compound per concentration per time point were used. At the end of each of the incubation periods, medium was transferred from each well to a microcentrifuge tube and was frozen at -80 ºC. Cells were then removed from each well with 250 µl of trypsin (as above), neutralized with medium, and counted, as described below. After counting, cellular suspensions were transferred to microcentrifuge tubes and frozen at -80 ºC to be used for various toxicological and biochemical assays, as described below.

III. Cell Count/Viability

Cellular suspensions and 0.4% trypan blue/medium solutions were formed using a dilution factor of 1/5 cell suspension/trypan blue. Viable cells were determined 5 minutes later by counting the samples on a hemocytometer, using a light microscope.

IV. Superoxide Anion Production

Determination of cellular levels of superoxide anion was performed according to the method of Babior et al. (1973), and was based on cytochrome c reduction. The 1.5 mL reaction mixture contained 50 µl cellular suspension and 0.05 mM cytochrome c in phosphate buffered saline, adjusted to pH 7.2. Reaction mixtures were incubated for 15 minutes at 37°C, and reaction tubes were then inserted into crushed ice to stop the reaction. Absorbance was determined at 550 nm, using a BioTek uQuant spectrophotometer (BioTek U.S., Winooski, VT). Absorbance values were converted to nmol of cytochrome c reduced/min/ 10^6, using the extinction coefficient 2.1 x $l0^4$ M^{-1} cm^{-1}.

V. Superoxide Dismutase (SOD) Activity

SOD was determined according to the method of Marklund and Marklund (1974). This method is based on the inhibition of autooxidation of pyrogallol by SOD. The reaction mixtures contained 200 mL of the cellular suspension, 750 mL tri cacodylic buffer (50 mM Tris–HCl, 50 mM cacodylic acid and 1 mM EDTA, pH 8.2) and 250 mL of 0.5 mM pyrogallol. Absorbances of the mixtures were recorded at 420 nm every 30 seconds over a period of 3 minutes and changes in the rates of these absorbances were then converted into units of SOD activity/10^6 cells, where one unit is equivalent to the quantity of SOD that is required to produce 50% inhibition of pyrogallol autooxidation.

V. Lipid Peroxidation Determination

Lipid peroxidation was determined using the colorimetric method of Uchiyama and Mihara (1978) by measuring the formation of thiobarbituric acid-reactive substances (TBARS). The reaction mixtures contained 0.25 mL of medium, 0.5 mL of 0.6% thiobarbituric acid and 1.5 mL of 1.0% phosphoric acid. The mixture tubes were then heated to boiling for 45 min and thibarbituric adducts were extracted with 2 mL of 1-butanol. Absorbances of the 1-butanol layer were determined at 535 nm, using a spectrophotometer, and TBARS concentrations were determined using a molar absorptivity constant of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Results

Results for cellular viability in response to DCA are shown in figure 1. Except for 4100 ppm of DCA that resulted in significant decline in cellular viability, the other compound concentrations showed no significant effects on that biomarker, after 24 h of incubation, when compared with the control. However, significant and concentration-dependent decreases in cellular viability were produced by the compound after 48-72 h of incubation.

Results for cellular viability following incubation of cells with TCA are shown in figure 2. Cells exposed to 1540 and 4100 ppm of TCA showed significant drop in viability in comparison to controls after 24 h incubation. After 48-72 h of incubation, all TCA concentrations tested resulted in significant and concentration-dependent decreases in viability.

Figure 3 displays the effects of DCA on SA production in cells after 24, 48, and 72 h. Significant SA production was observed with DCA concentration of 770 ppm at the three tested time points, when compared with the corresponding controls at those time points. DCA concentration of 1540 also resulted in significant SA production at the 24 and 48 h time points, but compound's concentration of 4100 ppm did not result in any significant change in SA production, 24 h after incubation when compared with the corresponding controls at the different time points. The effects of 1540 ppm on SA production after 72 h of incubation and that of 4100 after 48-72 h of incubation could not be determined because of the high rate of cellular death and unavailability of enough cells to be tested.

SA production at 24, 48, and 72 h of TCA exposure is shown in figure 4. At 24 h time point, TCA concentrations of 1540 and 4100 ppm resulted in significant SA production when compared with the control. TCA concentration of 770 ppm also resulted in significant SA

production but only after 48-72 h of incubation when compared with the corresponding controls. As with DCA, TCA concentrations of 1540 ppm and 4100 ppm resulted in significant cellular death after 48, and 48-72 h incubation for 1540 and 4100 ppm, respectively, that insufficient cells were present to conduct the test for SA production.

Superoxide dismutase (SOD) activity was measured after exposure to different concentrations of DCA at 24, 48, and 72 h (figure 5). At 24 h, SOD activity was increased only in response to 4100 ppm, when compared with the corresponding control. However, SOD activity was significantly increased in response to DCA concentrations of 770-1540 ppm and 770 ppm after respectively, 48 and 72 h of incubation when compared with the corresponding controls at those time points.

Changes in SOD activity in response to different concentrations of TCA at different times of incubation are shown in figure 6. At 24 h, SOD activity was significantly and dose-dependently increased in response to all doses, when compared with the control at that time point. After 48 h incubation, SOD activity was further dose-dependently increased, with approximately five-fold increase in activity observed at 1540 ppm, as compared with the corresponding control. SOD activity was also increased in response to 770 ppm of DCA after 72 h of incubation when compared with the corresponding control, but it was not significantly different from that observed with the same concentration at the immediately earlier time point (48 h).

As with SA production that could not be determined in response to certain DCA and TCA concentrations at certain time points because of the high rates of cellular death, SOD activity couldn't be determined in cells at those same concentrations and time points.

The effects of different concentrations and different times of incubation on the induction of LP by DCA are shown in figure 7. Significant increases in LP production were observed with concentrations of 4100 and 770 ppm, after 24 and 72 h of incubation, respectively, as compared with the corresponding controls on those time points. LP production in response to different TCA concentrations at different time points is shown in figure 8. After 24 h incubation, significant increase in TBARS formation was only observed with a TCA concentration of 4100 ppm when compared with the corresponding control. After 48 and 72 h of incubation, significant increases in TBARS formation were observed with 1540 and 770 ppm, respectively, as compared with the corresponding controls. Although LP production was determined in media, the high cell death produced in response to 1540 ppm and 4100 ppm at the 48 and 72 h, respectively, produced very unreliable results and could not be re-produced. Therefore, data for those concentrations/ time points were not shown.

Table 1 shows the statistical analysis for comparison between the effects of DCA and TCA on each biomarker at various time points. Data for each biomarker in response to each compound at a certain time point was pooled and compared with the corresponding pooled data for the other compound at the same time point using single factor ANOVA. While the effects of TCA on cellular viability and SOD activity during the 24-48 h period, as well as on LP production during the 24 h period were significantly greater than those of DCA, DCA effects on SA production during the 24-48 h periods were greater than those of TCA. No significant differences were between the effects of DCA and TCA on LP production at the 48 h period, and on all of the tested biomarkers in the 72 h period were observed.

Table 1. The calculated p-values for comparisons between the effects of DCA and TCA, using single factor ANOVA. Values < 0.05 indicate significant differences between DCA and TCA, with t and d denoting significantly greater effects produced by TCA and DCA, respectively, when the compounds compared with each other.

Biomarkers	**p-value**		
	24h	48h	72h
Cellular Viability	0.000172^{t}	0.010361^{t}	0.463315
SA	0.007405^{d}	0.002439^{d}	0.179647
SOD Activity	0.000335^{t}	0.008154^{t}	0.206333
LP	0.033883^{t}	0.060206	0.353897

Discussion

The AML-12 (alpha mouse liver 12) cell line was originally established at ATCC from mouse hepatocytes (CD1 strain, line MT42) transgenic for human TGF alpha. These cells exhibit typical hepatocyte features such as peroxisomes and bile canalicular like structures, and were therefore an appropriate *in vitro* model for studying the effects of DCA and TCA on the liver.

Results show significant SA production in response to DCA and TCA. Free radicals that are transiently generated from certain xenobiotics are known to be re-oxidized by oxygen, generating SA (Mason, 1982; Kappus, 1981). DCA and TCA metabolism involves generation of free radicals through reductive dechlorination pathways (Larson and Bull, 1992), and that those free radicals may have contributed to SA overproduction. Previous studies in mice showed that SA production in the hepatic tissues is associated with acute and long term exposure to DCA and TCA (Hassoun and Dey, 2008; Hassoun and Cearfoss, 2010), and therefore the results of the study are in line with *in vivo* studies.

The results show that DCA and TCA can induce concentration- and time-dependent decreases in cellular viability (increases in cellular death), that were associated with significantly higher levels of SA. However, SA levels underwent significant decline with increasing the exposure time to DCA, and variable very small changes with increasing the exposure time to TCA. These findings suggest that earlier production of SA has significant effects on the cells that lead to significantly greater cellular death with time, and may also indicate that some events are occurring between the time of SA production and the production of cellular death. SOD is an antioxidant enzyme that results in SA dismutation and its conversion to H_2O_2 (Rice-Evans, 1995), and the results indicate significant and time-dependent increases in SOD activity, suggesting significant SA dismutation by SOD, in response to the compounds. Hence, this event

may lead to significant H_2O_2 production that may have also contributed to the observed increase in cellular death with time. Since SA production in response to certain concentrations of the compounds was also associated with the induction of significant cellular death at the earlier time points, it is suggested that cellular death at earlier time points is mainly contributed by SA, while death occurring at a later time point is contributed by both SA and H_2O_2. These suggestions are in line with the conclusions of previous studies indicating the significant contribution of SA/H_2O_2 to the hepatotoxicity of DCA and TCA in mice after acute and long term exposure (Hassoun and Dey, 2008; Hassoun and Cearfoss, 2010; Hassoun and Cearfoss, 2011). This may also contribute to the observed increase in cellular death that is associated with increasing the compounds' concentrations. Although both compounds resulted in significant increases in SOD activity, significantly greater induction was observed with TCA as compared with DCA during the 24-48 h of exposure, suggesting a more significant contribution of this H_2O_2 to TCA- than DCA-induced cellular toxicity. However, the contribution of H_2O_2 to the DCA-induced cellular toxicity should not be ignored here, but it appears that SA plays a more significant role in DCA- than TCA-induced cell death, since DCA effects on the production of this biomarker during the 24-48 h of exposure were significantly greater than those of TCA.

In general, DCA and TCA resulted in concentration- and time-dependent increases in LP production. Previous studies indicated production of this biomarker in the hepatic tissues of mice after acute and long term exposure to the compounds (Larson and Bull, 1992; Hassoun and Dey, 2008; Hassoun and Cearfoss, 2010), and therefore our *in vitro* results are in line with the *in vivo* ones. ROS can attack the lipid layers of cellular membranes, resulting in LP (Rice-Evans, 1995). Hence the observed increases in LP production in the AML-12 cell cultures are associated with the SA/H_2O_2 over production in response to DCA and TCA. Also, Larson and Bull (1992) have

indicated a TCA pathway of metabolism that involves a one-electron reduction and hemolytic cleavage catalyzed by CYP-450, forming a dichloroacetyl radical that may abstract a hydrogen atom from lipids yielding DCA. Therefore, this mechanism may have also contributed to LP production by TCA, and may be confirmed by the observation that DCA induces significantly greater SA levels than TCA during the 24-48 h, but LP production by the compounds tend to reach similar levels with increasing the exposure time beyond 24 h. This can be also confirmed by the results of previous *in vivo* studies showing differences in LP production by TCA and DCA after 4-weeks of exposure, but the levels of induction of this biomarker by both compounds were similar after 13 weeks of exposure (Hassoun and Cearfoss, 2010)

DCA and TCA were previously shown to induce long term hepatotoxic and hepatocarcinogenic effects in mice (Daniel et al., 1992; DeAngelo et al., 1991; Herren-Freund et al. 1987), and that these effects were recently found to be associated with induction of various biomarkers of oxidative stress (Hassoun and Cearfoss, 2010; Hassoun and Cearfoss, 2011). However, there are several haloacetates other than DCA and TCA, as well as mixtures of haloacetates that still need to be tested. If those compounds and mixtures were to be tested in mice, as done with DCA and TCA, they will be challenged with several issues including ethics, associated with the use of large number of animals, cost, and length of time required to complete those studies. Therefore, this *in vitro* study was designed as an alternative to those done *in vivo*, and we primarily used it to test the effects of DCA and TCA in order to be able to compare the results with those observed *in vivo*. First, we show that the compounds can induce the production of various biomarkers of oxidative stress that are associated with hepatic cell death, similar to the induction of oxidative stress in the hepatic tissues which is associated with hepatotoxicity/hepatocarcinogenicity (Hassoun and Cearfoss, 2010). Second, it has been

previously suggested that earlier induction of various biomarkers of oxidative stress can lead to a later production of hepatotoxicity (Hassoun and Cearfoss, 2010) and this same suggestion can be applied to the observations of this study, as discussed above. Third, in the previous studies, SA was suggested to be the main contributor to DCA-induced hepatotoxicity, while SA/H_2O_2 were suggested to contribute to TCA-induced hepatotoxicity (Hassoun and Cearfoss, 2010), a conclusion similar to what is suggested here. Fourth, LP production was shown to be similarly induced by DCA and TCA in an *in vivo* system after long term exposure (Hassoun and Cearfoss, 2010) and is shown here that the levels of induction of this biomarker by the two compounds in the AML-12 cells tend to equalize with increasing time of exposure. However, while *in vivo* studies demonstrated the more hepatotoxic potential of DCA as compared with TCA (DeAngelo et al., 1989; Hassoun and Cearfoss, 2010), TCA appears in this study to be more toxic to cells than DCA during the 24-48 h periods of exposure. The results also show that the compounds become equitoxic with increasing the time of exposure. Hence, further studies to investigate the mechanism behind these differences are required.

When these studies were initiated, several trials were made with various concentrations (calculated as mg/mL) of the compounds, until the concentrations indicated in this study were proven to be effective/toxic. However, when these concentrations were converted to ppm (mg/l) for comparison purposes with the *in vivo* ppm (in terms of mg/kg), they were found to be 10 times greater than the tested *in vivo* doses ranging between 77-410 mg/kg/day (77-410 ppm/day). Those doses were found to correspond to the range of threshold-maximal *in vivo* doses of the compounds that produce hepatotoxicity/ hepatocarcinogenicity, as well as to the range of doses that is associated with significant production of oxidative stress in hepatic tissues (DeAngelo et al., 1989; Hassoun and Cearfoss, 2010). For the purpose of risk assessment, it is well known in

toxicology that the *in vivo* tested doses in animals are extrapolated to the No Adverse Effect Level (NOAEL), and that the NOAEL must be divided by uncertainty factor, which is usually 100, before it is extrapolated to humans. However, if the studies are performed *in vitro,* the uncertainty factor must be at least a one fold greater than the one used for the *in vivo* animals studies, i.e., 1000. Therefore, the effective DCA and TCA concentrations in this study are in line with the *in vivo* doses and are also in line with the factors used for the purpose of risk assessment. Also, "Reduce, Refine, Replace" (RRR) is a national and international ultimate goal for replacing animal studies with alternative systems, and the system suggested in this study can be an appropriate one for testing the effects of different haloacetates.

In summary, exposure of AML-12 cells to DCA and TCA at concentrations that are 10 times the range of threshold-maximal hepatocarcinogenic/ hepatotoxic doses of the compounds associated with the induction of various levels of oxidative stress in the hepatic tissues of mice result in concentration- and time-dependent increases in biomarkers of oxidative stress that can lead to cellular toxicity and death.

Recommendations for Future Studies

1. The results of this preliminary study show great differences in the effects produced by DCA and TCA at the tested concentrations and time points. Therefore, other concentrations and time points in between the tested ones should be tried to give a better idea about the trends of increases and decreases of effects with different times and concentrations, i.e., better view of concentration- and time-dependent responses.

2. Several other haloacetates that are formed with DCA and TCA during the process of water chlorination may be tested in this same system, and the effects can be compared with those of DCA and TCA.

3. Exposure to mixtures of contaminants is today's problem in toxicology, and regulatory agencies are mostly interested in that for regulatory purposes. This system may be used as an appropriate model to test the effects of unlimited combinations of haloacetates, which will save a lot of the cost and time associated with conducting those tests in animals. Also, the studies will cover the ethical issue that is always associated with any toxicity studies done on animals.

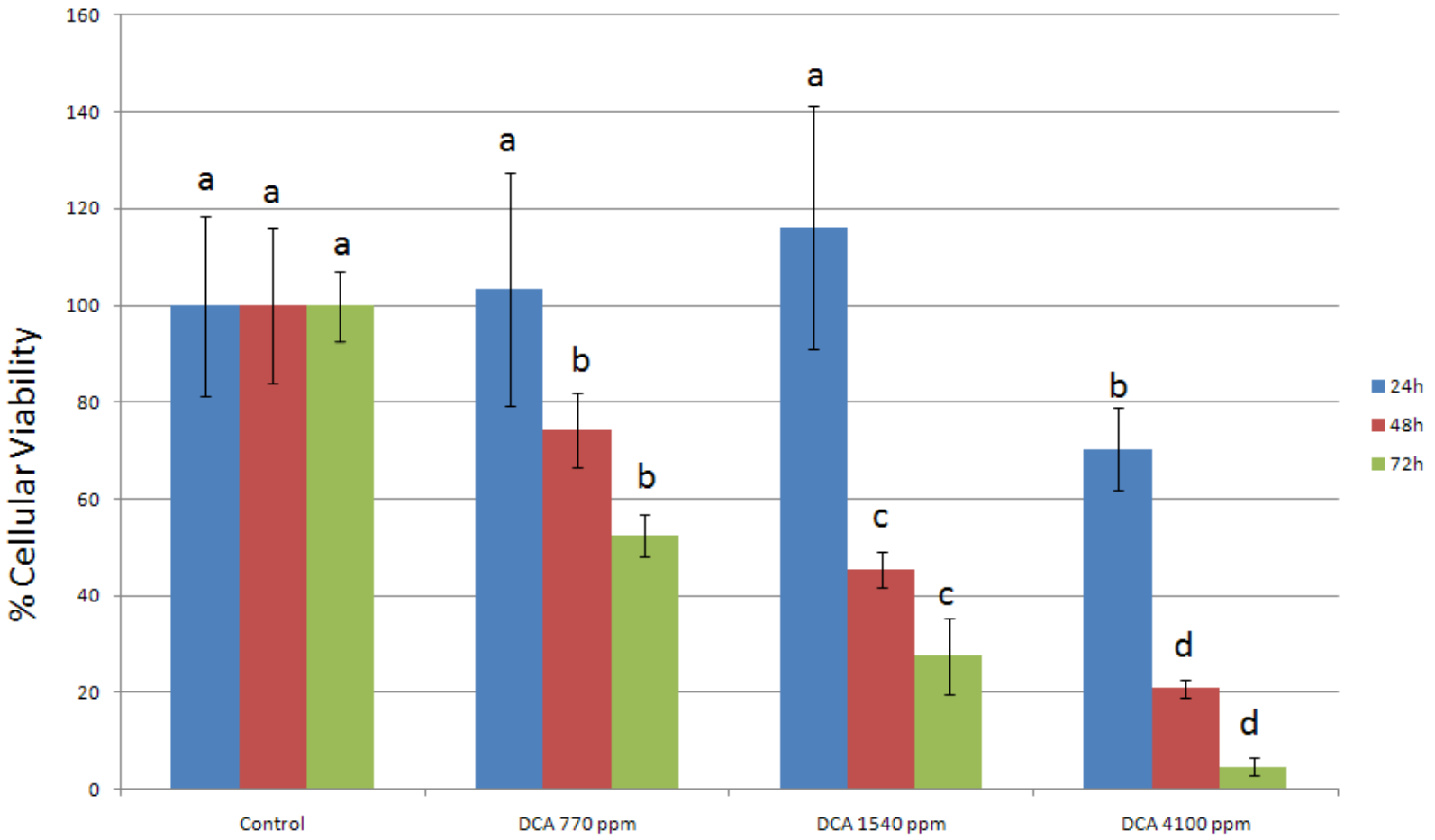


Figure 1. The concentration-response curves for the effect of DCA on cellular viability determined by trypan blue staining. Each point represents the mean ± SD. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

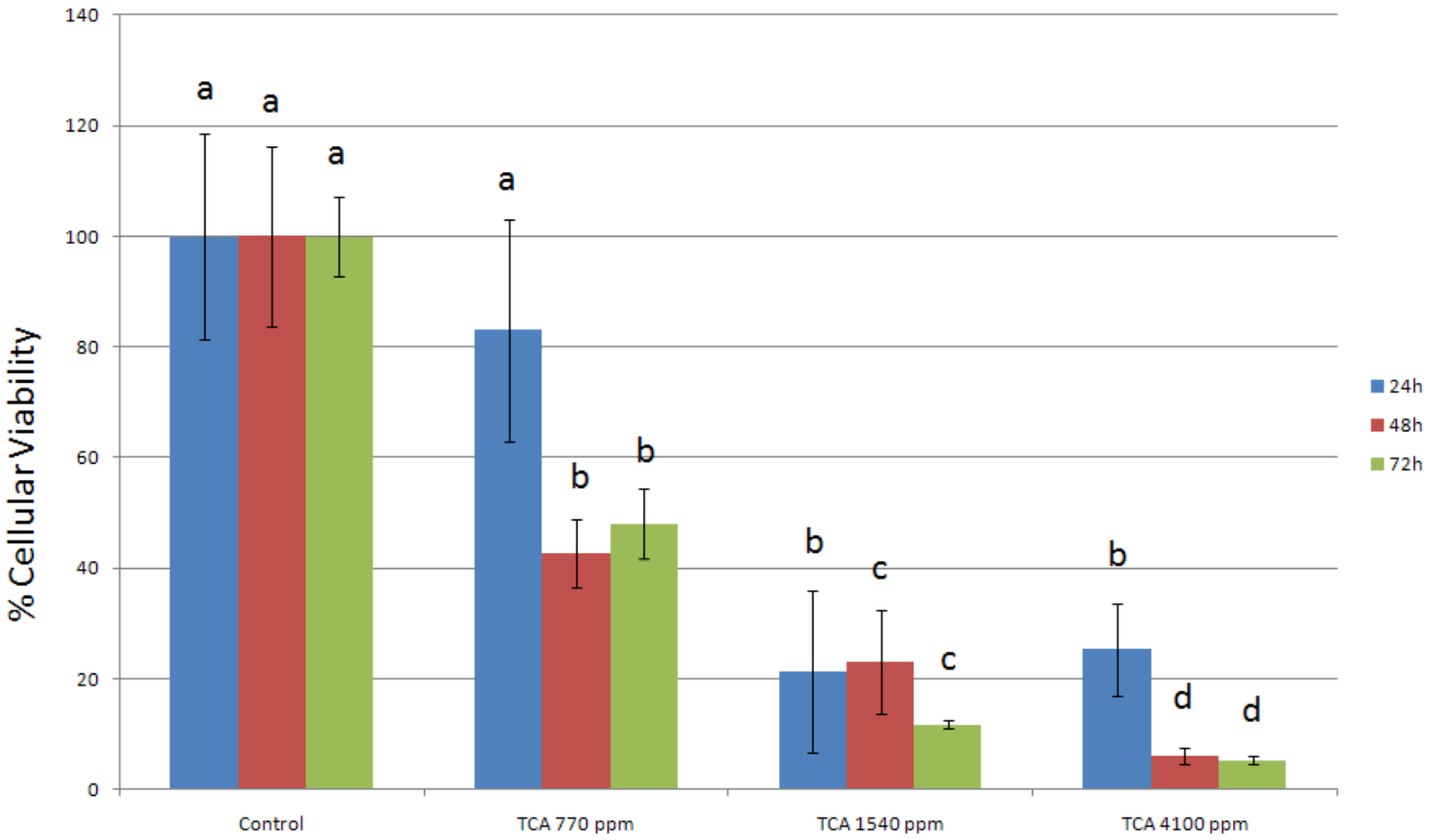


Figure 2. The concentration-response curves for the effect of TCA on cellular viability determined by trypan blue staining. Each point represents the mean ± SD. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

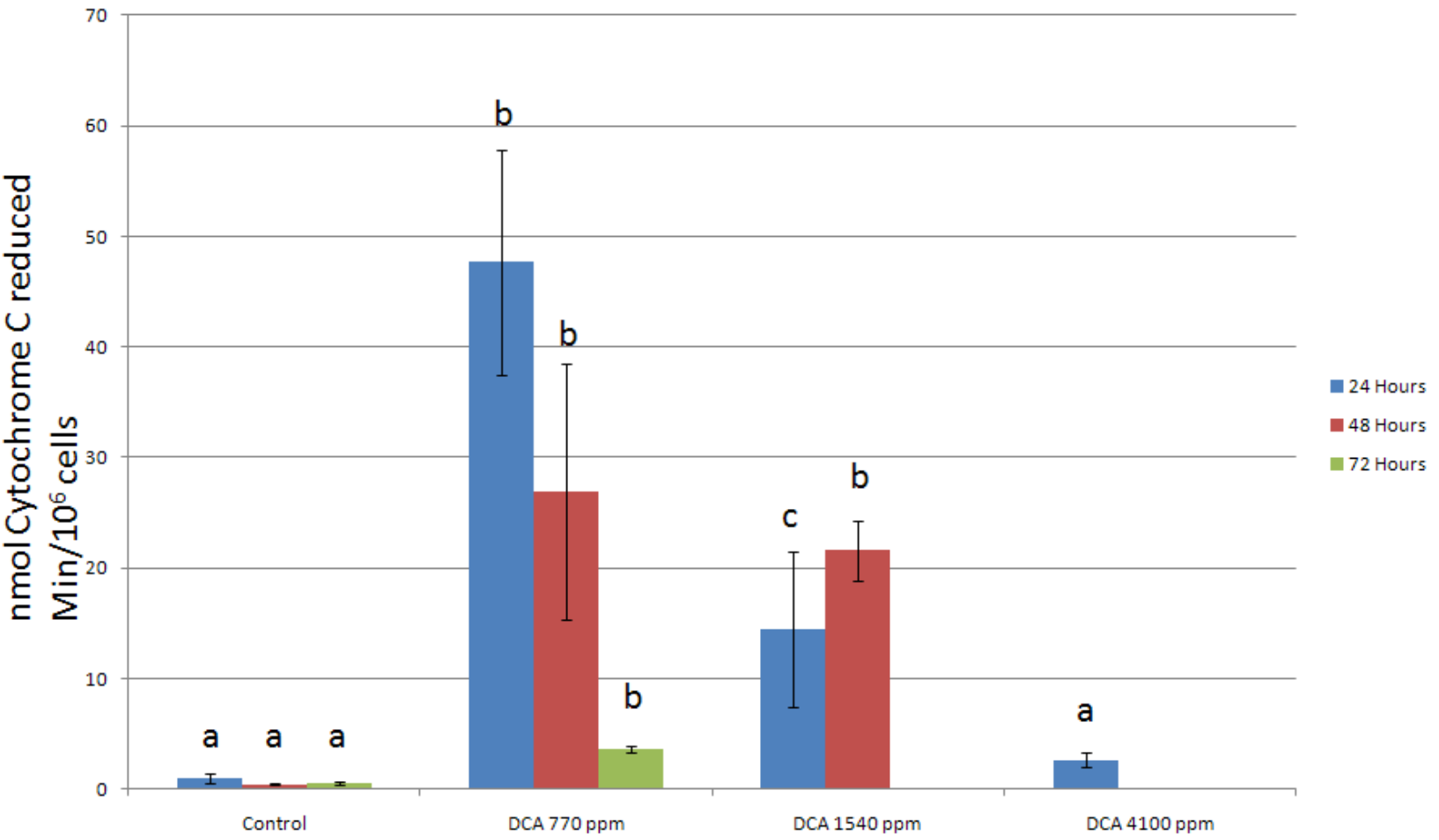


Figure 3. The concentration-response curves for the effect of DCA on SA production as determined by cytochrome C reduced/min/10^6 cells. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

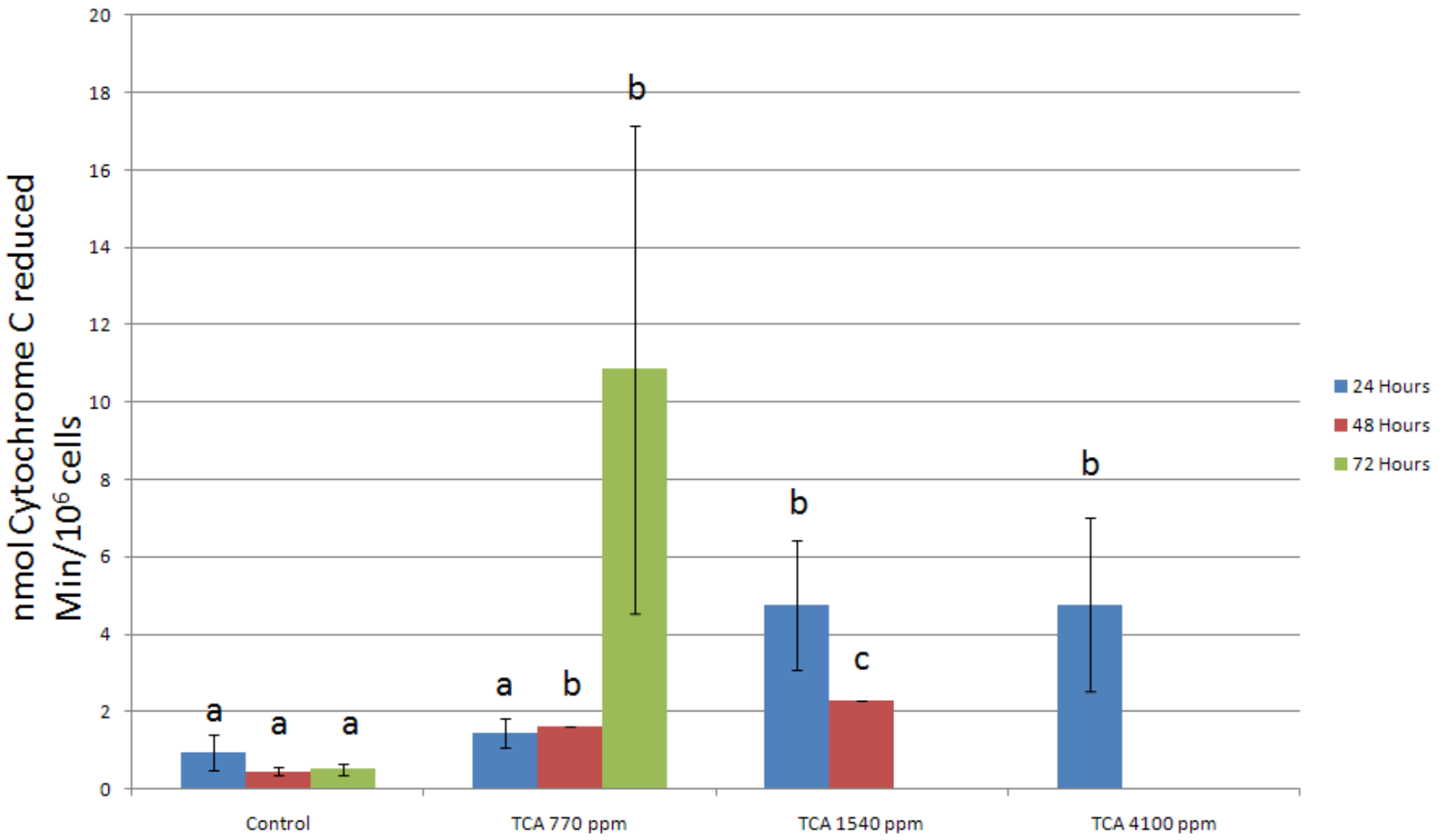


Figure 4. The concentration-response curves for the effect of TCA on SA production as determined by cytochrome C reduced/min/10^6 cells. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

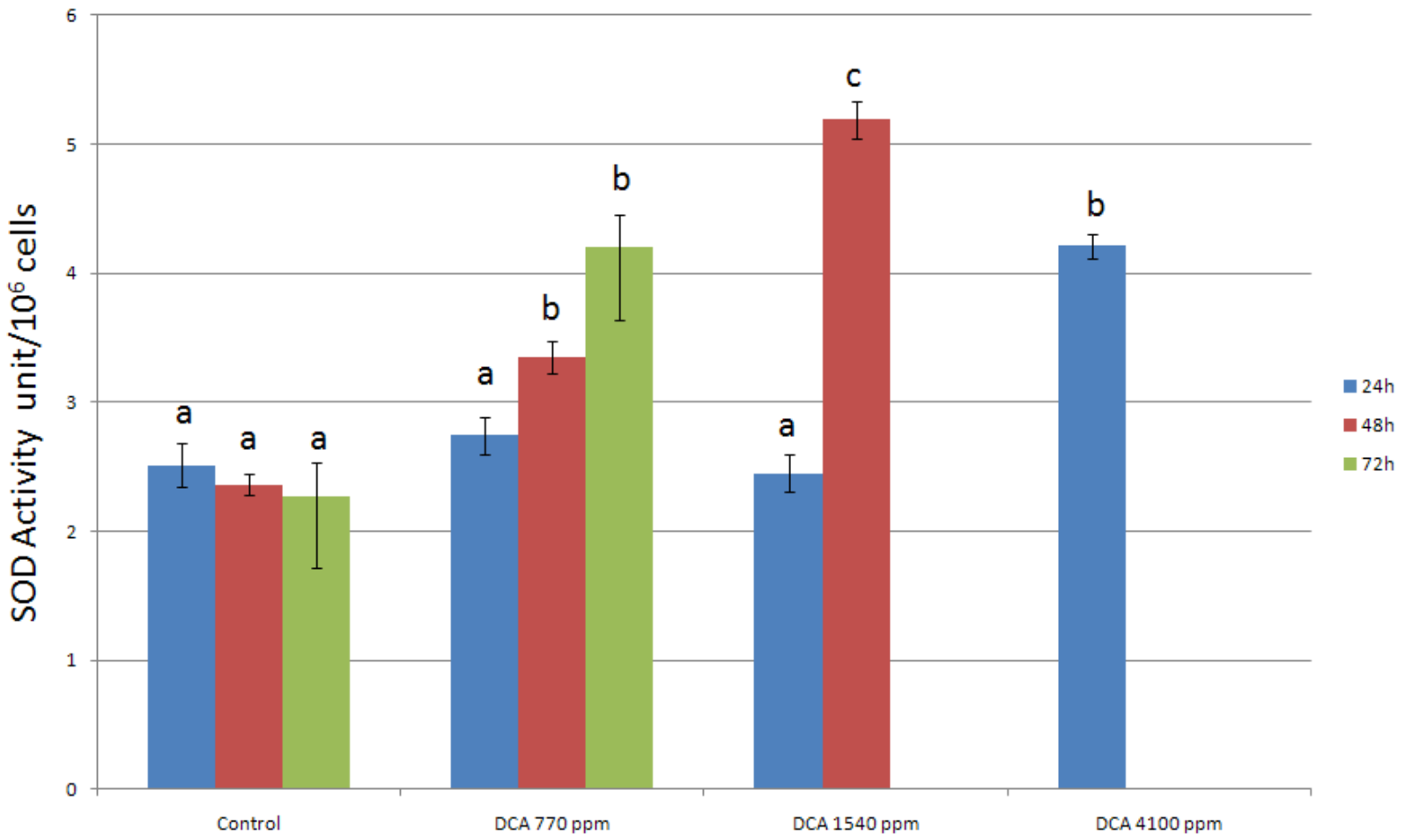


Figure 5. The concentration-response curves for the effect of DCA on SOD activity as determined by inhibition of pyrogallol/min/10^6 cells. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

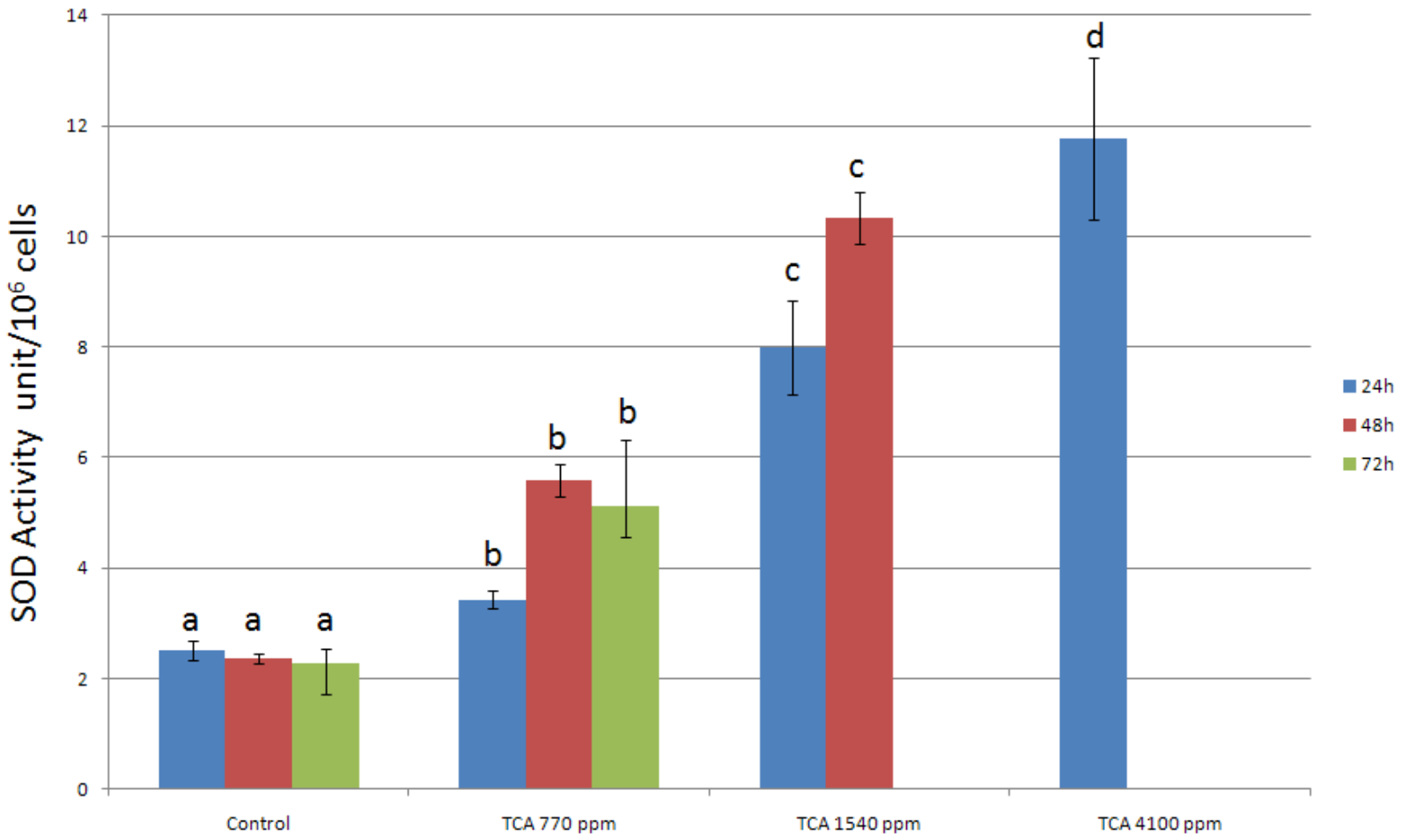


Figure 6. The concentration-response curves for the effect of TCA on SOD activity as determined by inhibition of pyrogallol/min/10^6 cells. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

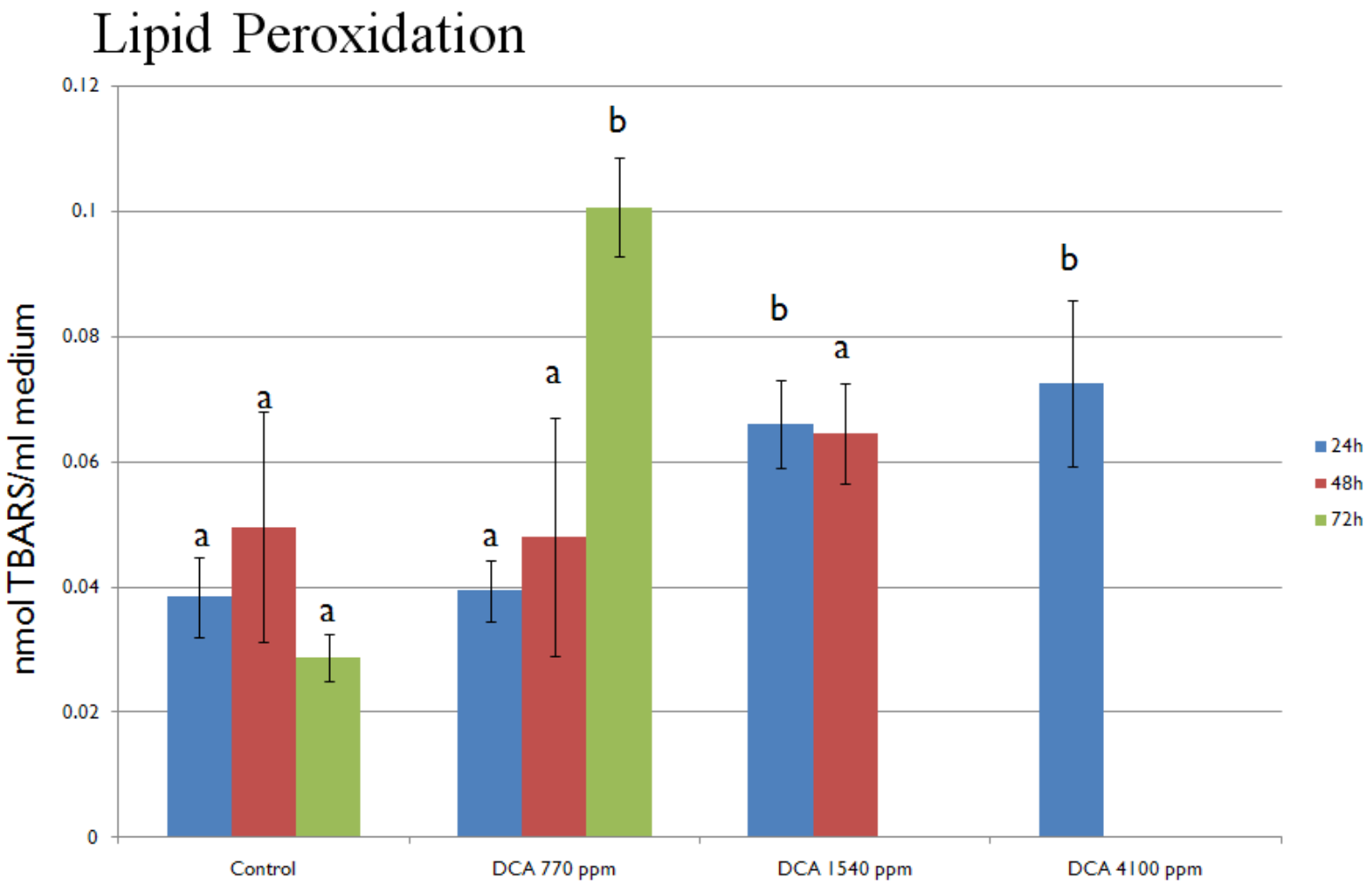


Figure 7. The concentration-response curves for the effect of DCA on LP as determined by formation of TBARS/ml medium. Each point represents the mean ± SD. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

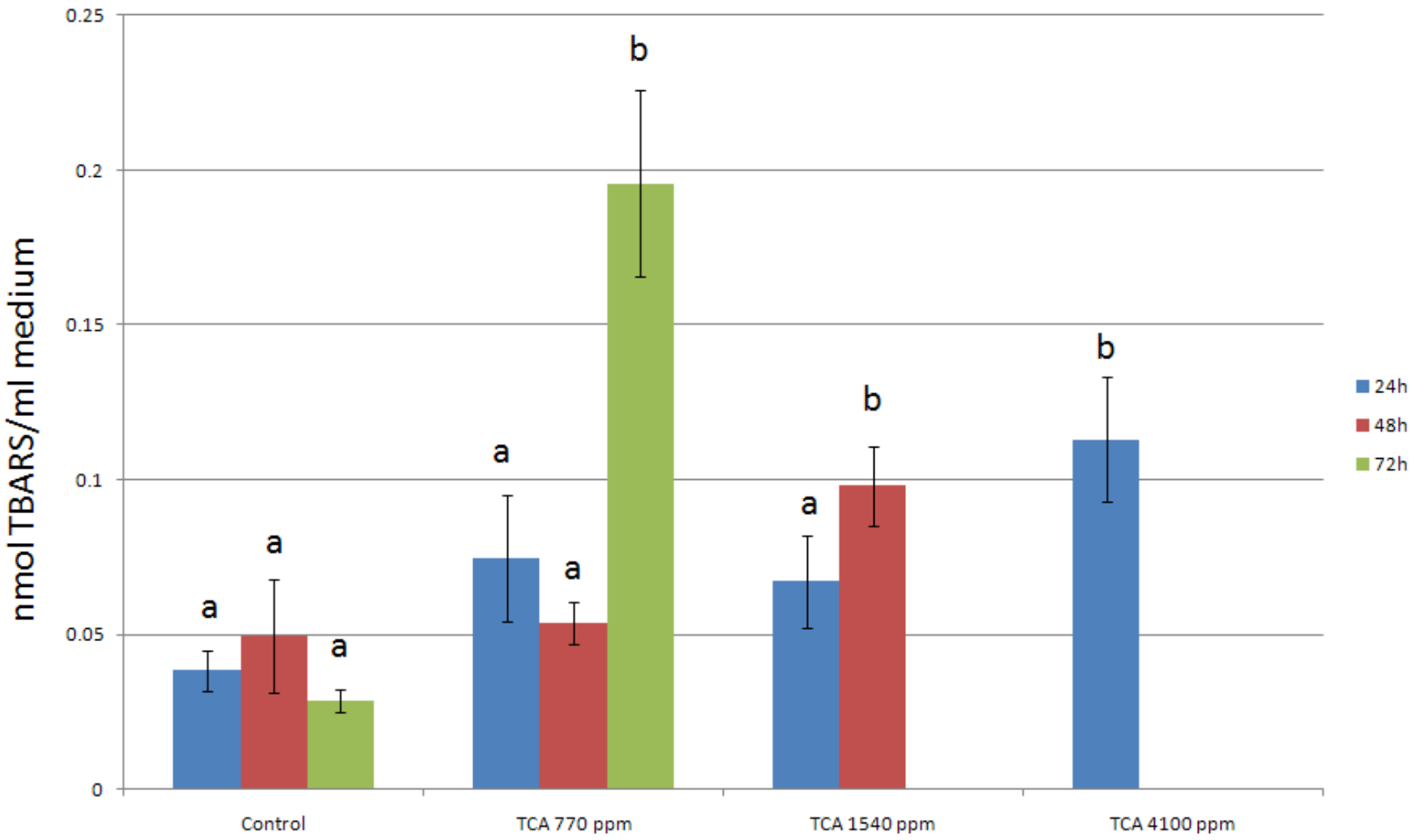


Figure 8. The concentration-response curves for the effect of TCA on LP as determined by formation of TBARS/ml medium. Each point represents the mean ± SD. Values within each time point with different superscripts are significantly different, using single factor ANOVA with $p<0.05$.

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