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The interaction between dichloroacetate, trichloroacetate and acetaminophen : effects on oxidative stress induction in AML 12 cells

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The Interaction Between Dichloroacetate, Trichloroacetate and Acetaminophen: Effects on Oxidative Stress Induction in AML 12 Cells

by

Mohammed Hasan Abdulkareem

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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Water chlorination process results in the formation of different haloacetate by- products, such as dichloroacetate (DCA) and trichloroacetate (TCA). Those compounds were previously found to be hepatotoxic and hepatocarcinogenic in rodents, and oxidative stress (OS) plays a role in that. Acetaminophen is a widely used analgesic and antipyretic, and doses above the therapeutic level are known to result in liver failure and hepatic damage. Therefore, we proposed a possible increase in induction of liver cells damage after exposure to mixtures of DCA, TCA and acetaminophen. To test this hypothesis, we performed *in vitro* studies using alpha mouse liver 12 (AML 12) cells as a model to screen the effects of mixtures of the compounds. Individual compounds and different mixtures of the compounds were incubated with the cells for 48 hours, and cellular viability and various biomarkers of OS production, including superoxide anions (SA), advanced oxidation protein products (AOPP), nitric oxide (NO) and lipid peroxidation were determined. DCA and TCA were tested at concentrations of 770 and 500 ppm, respectively, since they were previously shown to result in 25% cell death. A concentration of 755.8 ppm of

acetaminophen was found in this study to produce 25 % decrease in cellular viability, and was therefore used in the tested mixtures. Three binary mixtures, as well as a mixture of the three compounds were tested. All of the binary mixtures and the 3-compound mixture resulted in additive effects on the reduction in cellular viability. However, the net effects of the binary mixtures on the production of various biomarkers of OS was variable and ranged from less than additive to additive effect, but those of the 3-compound mixture on those biomarkers were all significant increases that ranged from additive to greater than additive effects. It is concluded that the compounds induce significantly greater effects on AML 12 cells when they exist in the form of a 3-compound mixture and a focus should therefore be given in the future to studying different mixture compositions containing the three compounds, both *in vitro and in vivo.*

For my wife, baby, parents and great family

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Chapter 1

Introduction

1.1 Water Chlorination

Treatment of water to make it safe for public use has been one of the major concerns in the twentieth century. During the disinfection process, chlorine is added to water to destroy the pathogens that are responsible for causing major diseases such as cholera, typhoid fever and hepatitis A (1). Chlorine is usually added at the lowest safe concentration required to kill the germs (2). In fact, chlorine is added as gaseous form (Cl_2) or as liquid (sodium hypochlorite NaOCl) which reacts with water to produce the oxidizing agent, hypochlorus acid (3). However, reaction of chlorine with the organic materials in surface water, mainly fulvic and humic acids was found to generate disinfection by products (DBPs) which include trichloroacetate (TCA) and dichloroacetate (DCA) (4). Also, TCA and DCA are significant metabolites that are formed *in vivo* , in animals and humans, from exposure to the industrial solvent trichloroethylene found as water contaminant in several geographical area (5).

1.2 Oxidative Stress (OS) and Free Radicals

Oxidative stress (OS) is referred to the imbalance between oxidation and antioxidation status of the cells in favor of oxidation (6). The imbalance in cell status leads

to over production of free radicals and reactive oxygen species (ROS) that attack the biological membrane (7), DNA, RNA, protein and other cellular macro-molecules (8). Normally, free radicals are generated from normal metabolic processes of endogenous compounds, or from external sources such as exposure to x-ray, ozone, and other xenobiotics. However, oxygen molecule can also generate oxygen free radicals or ROS such as hydrogen peroxide, superoxide anion and nitric oxide (9). Generation of ROS is associated with the production of many diseases and pathological conditions, such as aging (10), Alzheimer's disease (11), cardiovascular diseases (12), pulmonary inflammation (13), and Down's Syndrome (14). ROS are also capable of transmitting both extracellular and intracellular signals to the nucleus, and can be involved in the modulation of various enzymes such as protein kinases C, especially when they are present in low concentration (15). These protein kinases have a noticeable role in the regulation of cellular responses such as activation, proliferation and differentiation. Thus, OS is not only a cytotoxic condition , but it also plays a major role in the regulation of cellular functions (7).

1.3 Acetaminophen

Acetaminophen is a p-aminophenol derivative that is considered as the most well-known analgesic and antipyretic used around the world. Although the mechanism of its effect is not fully understood yet, but inhibition of nitric oxide pathway through several processes mediated by neurotransmitters such as N-methyl-D-aspartate and substance P have been suggested (16). Also, several studies have suggested that inhibition of prostaglandin synthesis in the CNS may be responsible for its antipyretic activity (17-19).Acetaminophen has a pH of 6 and a molecular weight of 151.16256 g/mol, and it is slightly soluble in water (20).

Acetaminophen at doses exceeding the therapeutic level is known to produce hepatotoxicity and liver damage. Studies in Dr. Gillette's laboratory in the 1970s were the first to report that acetaminophen is activated by cytochrome p450 to form the highly reactive toxic metabolite, *N*-acetyl-*P*-benzoquinone imine. This toxic metabolite depletes glutathione level, covalently binds with mitochondrial proteins, and contributes to acetaminophen major toxicity pathway (21, 22). HepaRG cell line was used as a model to study acetaminophen induced hepatotoxicity in human, where depletion of GSH, formation of protein adduct, mitochondrial dysfunction and lactate dehydrogenase release that were associated with mitochondrial reactive oxygen and reactive nitrogen formation have been reported (22, 23).

1.4 Dichloroacetate (DCA) and Trichloroacetate (TCA)

DCA is one of the important by products that are formed during the process of water chlorination and its concentration in the chlorinated water was found to be around 100 µg/L (24, 25). The molecular weight of the sodium form of the compound is 151g/mol and it has a good solubility in water, alcohol and ether with a pKa of 1.29. DCA is well absorbed from the gastrointestinal tract (GIT) of rodents and it undergoes biotransformation to form several metabolites, mainly gloxylate, glycolate and oxalate (26). About 40% of the absorbed DCA was found to be present as unbound form in the blood of rodents, and the compound has a dual excretion pathway: urinary and biliary (27, 28).

Trichloroacetate (TCA) is another water chlorination by product which as a sodium salt has a molecular weight of 185.37 g/mol and has high water solubility. TCA was found to be immediately absorbed from the GIT of rodents, and metabolized in the liver (29). Ninety percent of TCA was found to bind to albumin (30), and was also found to be

removed unchanged in rodents, mainly in urine, with a small percentage excreted in bile (28).

Although DCA is not largely used as a therapeutic agent due to its observed toxic effects in labrotary animals, it was found to have some benefits in the treatment of acute lactic acidosis, diabetes, and hypercholostermia (24). TCA on the other hand had a wide history of use for skin treatment as a superficial peeling agent of acne scars(31). The compound is also used in industry as pickling agent for surface treatment of metals and as antiseptic and a reagent for the synthesis of different organic compounds. Human exposure to DCA and TCA results from consumption of the chlorinated drinking water or from the *in vivo* metabolism of trichloroethylene (TCE), that is present as a water contaminant (32). The most notable effects of DCA and TCA are hepatotoxicity and hepatocarcinogenicity that have been seen particularly in B6C3F1 male mice after acute and long-term exposures (33-35)

1.5 Superoxide Anion (SA)

Superoxide anion (SA) is a ROS formed by the reduction of an oxygen molecule through enzymatic pathway by xanthin oxidase or NADPH oxidase, or non-enzymatic pathways through redox reactive compounds such as semi-ubiquinone in the mitochondria (36). SA can be dismutated to another ROS, which is hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD). Hydrogen peroxide can be detoxified and converted to water molecules by the action of two antioxidant enzymes, glutathione peroxidase(8) and catalase (37). However, hydrogen peroxide can be converted to the highly toxic hydroxyl radical by the Fenton reaction (38).

1.6 Advanced Oxidation Protein Products (AOPPs)

AOPPs are derived from the oxidation of proteins by the ROS (39, 40). AOPPs were found to accumulate in patients with different diseases including, renal and coronary artery diseases(39, 41), cancer (42), and rheumatoid arthritis (43), and have been suggested to be as biomarkers for the production of these diseases. Most AOPPs are formed due to the release of myeloperoxidase (MPO) from activated phagocytes. MPO, the heme enzyme, catalyze the reaction between chloride ion and hydrogen peroxide to form a powerful oxidizing agent, hypochlorous acid (HOCl).Furthermore, MPO utilized H2O2 and $NO2^{-}$ to form microbicidal oxidant which can nitrate phenolic compounds and proteins (44).

1.7 Nitric Oxide (NO)

NO is a free radical that is produced by the action of nitric oxide synthase (NOS), which is a heme-containing monooxygenase enzyme (45). NOS catalyzes the degradation of L-arginine to L-citrulline to NO in the presence of NADPH and oxygen (46). NO plays an important role in major body functions, such as vasodilation, platelet function, blood flow and mitochondrial respiration (47). NO effects are found to be initially mediated by the activation of cGMP that is known as a vasodilation mediator and an inhibitor of platelet aggregation and can also prevent vascular smooth muscle cell growth. However, NO may also produce harmful biological effects through a non-cGMP pathway by forming high affinity nitroso compounds that interact with haem-containing proteins, such as oxyhemoglobin (48). Further, NO effects can be either direct or indirect, depending on the concentration. The direct effect is induced through binding of NO with the biological targets when NO concentration is less than 1μM , while the indirect effect is induced when

the concentration of NO is more than 1μM and it results from binding of NO with SA or oxygen , producing reactive nitric oxide species (RNOS) (49).

1.8 Lipid Peroxidation

Lipid peroxidation is one of the most important mechanisms in the pathogenesis of numerous diseases in humans. The process of lipid peroxidation is generated by the action of ROS, such as H_2O_2 and the hydroxyl radical. The ROS attack fatty acid molecules of the cellular membranes promoting self-propagation chain reaction (50). The oxidized lipid is more stable than the parent ROS, and therefore can be distributed from the site of their location and cause damage at a remote location. Increased formation of lipid peroxides were observed in many pathological condition such as rheumatoid arthritis, ischemia, Alzheimer disease, and immunological disorder (51).

1.9 OS in Acetaminophen -Induced Hepatotoxicity and Cell Death

The toxicity of acetaminophen is mediated by the highly reactive metabolite *N*-acetyl-*P*-benzoquinone imine, which is considered as an electrophile and oxidizing agent that binds covalently with critical proteins, or generates oxidative damage (52). Acetaminophen toxicity was shown to occur after passing of the compound through two phases; a metabolic phase and an oxidative phase. The metabolic phase was found to be associated with increased glutathione depletion and an increase in the formation of oxidized glutathione (GSSG). The oxidative phase was found to involve increased OS production and loss of mitochondrial membrane potential (MPT) (53, 54).

Significant cell death was shown to be induced in mouse hepatocytes incubated with 1mM of acetaminophen for 24 hours (53, 55). Furthermore, necrosis and cell damage were observed in the hepatocytes of mice exposed to toxic doses of acetaminophen (56).

1.10 OS in DCA- and TCA- Induced Hepatotoxicity and Hepatocarcinogenecity

The abilities of DCA and TCA to induce OS were previously assessed by the production of lipid peroxidation , DNA damage and production of superoxide anion in the livers of mice treated with either acute doses of the compounds (35) or subchronic doses of the compounds that ranged from non hepatotoxic/ hepatocarcinogenic to those causing maximum hepatocarcinogenicity (35, 57). Lipid peroxidation, determined as increased formation of thiobarbuturic acid reactive substances (TBARS), and production of DNA damage assessed by 8-hydroxydeoxuguanosine (8-OHdG) formation, were aslo found to be induced in the livers of mice after acute administration of the compounds (58). Studies suggested that OS production may be considered as an early biomarker that is induced by DCA and TCA mixtures and can lead to long term hepatotoxic and hepatocarcinogenic effects (59).

Chapter 2

Objectives

1.Dichloroacetate (DCA) and trichloroacetate (TCA) are known to induce hepatotoxic and hepatocarcinogenic effects in rodents. Acetaminophen overdose was also proven to cause hepatotoxicity in *vivo*, in rodents and in humans. DCA and TCA are present in the drinking water as by- products of the water chlorination process, and acetaminophen is on top of the list of the analgesic and antipyretic products used around world. Consequently, the expected life time drinking of chlorinated water containing the chloroacetates together with the frequent use of acetaminophen by some individuals may alter the risk of acetaminophen hepatotoxicity. This study is a preliminary investigation of any possible interactions between the three compounds when they exist as mixtures.

2. Since in *vivo* studies are associated with high cost, time consumption, and ethical issues in using large number of laboratory animals, we conducted the studies *in vitro*, on AML-12 cells which are mouse liver cells. This model may help achieve the goal of replacing, refining, and reducing the use of animals, and at the same time may provide preliminary results that help initiate future studies with lower numbers of animals.

3.Oxidative stress was shown to play a role in the induction of hepatotoxicity by DCA, TCA and acetaminophen. In this study, we proposed roles for a number of biomarkers of

oxidative stress including SA, NO, AOPP and lipid peroxidation in the induction of liver cell toxicity and death.

Chapter 3

Materials and Methods

The chemicals used in the studies were purchased from Sigma Aldrich (St. Louis, MO), and they were of the highest available quality.

3.1 Alpha Mouse Liver-12(AML-12) cells

AML-12 cells were established from hepatocyte of CD1 strain male mouse that is transgenic for human (TGF-α). Under microscope, the cells are recognized by typical hepatocyte characteristics such as peroxisomes and obvious bile canalicular like structures. AML-12 cells express high level of serum mRNA and contain solely isozyme 5 for lactate dehydrogenase (60). Cells were cultured in Dulbecco's Modified Eagle-Ham medium, and 100 ml of fetal bovine serum(FBS) was added to 900 ml of the medium. The following were then added to the medium containing FBS: 0.005 mg/ml insulin, 0.005mg/ml transferrin, 40 ng/ml dexamethasone, and 5 ng/ml selenium. Cells were cultured in medium and were incubated for 48 hours at 37ºC in humidified incubator with 5% CO2. The medium was removed after incubation, and 2ml of trypsin was added to the flask and incubated for 10 minutes to detach the cells. Ten ml of medium was then added to each flask, and cellular suspensions were centrifuged for 10 minutes at 3000 rpm. The supernatants were removed and cells were re-suspended in medium of above described

composition. Cellular suspensions were added to culture dishes and were incubated overnight before they were treated.

3.2 Chemical Preparation and Cellular Treatment

Three chemicals were tested in the study, either alone or as mixtures: DCA, TCA, and acetaminophen. DCA and TCA solutions were prepared in the same medium used for cell culture and they were added at a volume of 1.5 ml to each dish containing 1.5 ml media (3ml total volume) to obtain final concentrations of 770 ppm, 500 ppm, respectively. Acetaminophen solutions were also prepared in the medium and were added to different dishes at (3779, 3023.2, 2267.4, 1511.6 and 755.8 ppm). The solutions were added at a volume of 1.5 ml to each culture dish containing 1.5 ml of medium (3ml total volume). Mixtures were also prepared and added at a volume of 1.5 ml to each culture dish that contained 1.5 ml medium (3ml total volume). The following mixtures were tested at the indicated final concentrations of the compounds:

- DCA 770 ppm + TCA 500 ppm
- DCA 770 ppm + Acetaminophen 755.8 ppm
- TCA 500 ppm + Acetaminophen 755.8 ppm
- DCA 770ppm + Acetaminophen 755.8 ppm + TCA 500 ppm

All dishes were incubated for 48 hours at 37ºC. After the incubation period, media were removed and transferred to Eppendorf tubes and were frozen at – 80Cº. 2 ml of fresh medium was added to each dish, and cells were detached from the culture dishes by scraping. Cellular suspensions were assayed for cellular viability as indicated below and were then transferred to Eppendorf tubes and frozen at -80Cº. The frozen media and

cellular suspensions were used for the determination of various biomarkers of OS as indicated below.

3.3 Determination of Cellular Viability

Cellular suspensions were mixed at a volume of 100 µl with an equal volume of 0.4% trypan blue solution that was prepared in phosphate buffer saline (PBS). The mixture was placed on each side of Luna cells counting slides of an automated digital cells counter device (logos biosynthesis™) and cell numbers were determined.

3.4 Determination of Superoxide Anion (SA)

Cytochrome c reduction method was used for SA determination (36). Twenty-five µl of cellular suspension were added to 1.5 ml of 45 nmole cytochrome *c* oxidase in PBS (PH 7.2). The reaction tubes were incubated for 15 minutes at 37ºC and they were then placed on crushed ice to stop the reaction. The absorbances were determined at 550 nm using Genesis 20 ® spectrophotometer (Rochester, NY). Absorbance of a blank containing only 1.5 ml of cytochrome *c* oxidase in PBS was also determined. The absorbance of each sample was converted to nmole of cytochrome *c* reduced/min by using the extinction coefficient of $2.1 \times 10^{-} M^{-1} cm^{-1}$.

3.5 Determination of Advanced Oxidation Protein Products (AOPPs)

AOPPs is a novel marker for OS and the concentrations were determined according to the method of Witko-Sarsat (39). Two hundred µl of the medium from different culture dishes were mixed with 800 µl of PBS contained 136.88 mM NaCl, 2.68 mM KCl. 8.1 mMNa_2HPO_4 and 1.47 mM KH_2PO_4 (PH 7.2). Then, 50 µl of 1.16 M KI and 100 µl of acetic acid were added to the mixtures and the absorbance of each sample was read at 340

nm. Blank containing PBS, KI and acetic acid was also prepared. The absorbances of samples and blank were determined in Genesis 20 spectrophotometer (Rochester, NY).

A standard curve was prepared using chloramine T solutions at concentrations of100, 80, 60, 40, 20, 10 and 7.5 µM. One ml of each concentration was added to a tube and was mixed with 50 µl of 1.16 M KI and 100 µl of acetic acid and the absorbance of each was read at 340 nm using Genesis 20® spectrophotometer (Rochester, NY). Figure 1 shows the AOPPs standard curve and the equation used to determine the AOPPs concentrations in the samples.

3.6 Determination of Nitric Oxide (NO)

NO concentration was determined using Greiss reagent(61). Greiss reagent was prepared by mixing equal volumes of two solutions: solution 1 contained 0.1% of N-(-naphthyl) ethylene diamine, and solution 2 contained 1% of sulfanilamide in 5% phosphoric acid. A volume of 100 µl of cell suspension was mixed with 650 µl of distilled water and 750 µl of Greiss reagent. The mixtures were incubated at 37ºC for 5 minutes and the reactions were stopped by placing the tubes in crushed ice. Absorbances were read at 543 nm, using Genesis 20 ® spectrophotometer (Rochester, NY). A standard curve was also prepared as follows:

Thirteen concentrations of sodium nitrate (10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.75, 0.5 and 0.25µM) were prepared from 10 µM stock solution, by serial dilutions. Then, 750 µl of each concentration of sodium nitrate was mixed with 750 µl of Greiss reagent and mixtures were incubated at 37ºC for 5 minutes and the reactions were then stopped by placing the tubes in crushed ice. Absorbances of the standard solutions were determined at 543 nm,

using Genesis 20® spectrophotometer (Rochester, NY). Figure 2 shows the standard curve, and the equation used to determine the concentrations in the samples.

3.7 Determination of Lipid Peroxidation

Lipid peroxidation was determined as production of TBARS, using the method of Uchiyama and Mihara (62). The reaction mixtures contained 250 µl of cellular suspension, 0.5 ml of 0.6% thiobarbituric acid, and 1.5 ml of 1% phosphoric acid. The mixture tubes were heated in boiling water for 45 minutes and were then allowed to cool at room temperature. Two ml of n-butanol was added to each sample tube for the extraction of the TBARS. The tubes were vortexed and then centrifuged for 10 minutes at 3000 rpm, and absorbances of the n-butanol layers were measured at 535 nm, using Genesis 20® spectrophotometer (Rochester, NY). Nmoles of TBARS were calculated using an extinction coefficient of $1.56*10^5\ M^{-1}Cm^{-1}$.

3.8 Statistical Analysis

Data are presented in each point of the figures as the mean $\pm$ SD of four cultures (dishes) / treatment, and were subjected to analysis of variance (single ANOVA). Sscheffe's method was used as a post hoc test, and a significance level of $p< 0.05$ was employed.

Chapter 4

Results

For testing the effects of DCA, TCA and acetaminophen mixtures on cellular viability, appropriate concentration of each compound was considered. Previous studies have determined the time and concentration effects of DCA and TCA on cellular viability (63), DCA and TCA concentrations used in this study were based on that. However, acetaminophen effect on cellular viability was determined in this study, and figure 3 shows the effects of different compound concentrations on cellular viability after 48 h of incubation. The figure shows significant and concentration-dependent decrease in cellular viability, with maximal decrease observed at concentrations ranging between 3779 to 755.8 ppm.

Figure 4 shows the effects on cellular viability when cells were treated with 770, 550, and 755.8 ppm of DCA, TCA, and acetaminophen, respectively, as well as with their mixtures at those concentrations. Significant reductions in cellular viabilities were observed in the DCA, TCA and acetaminophen treated cells when compared with the control. Figure 4 also shows the effects of mixtures of the compounds on cellular viabilities and also comparisons of their effects with those of the individual compounds composing them. Significant

reduction in cellular viabilities was observed in all of the binary mixtures, as well as the 3-compound mixture when compared with effects of the compounds composing them.

Figure 5 shows the effects of DCA, TCA, acetaminophen and their mixtures on SA production. Significant increases in SA production were observed with all of the individual compounds treatment when compared with the control. For the binary mixtures effects, DCA + TCA mixture showed a significant increase when compared with each of DCA and TCA effects, DCA + acetaminophen mixture effect was not significantly different from those of DCA and acetaminophen, and TCA + acetaminophen effect was significantly lower than those of TCA or acetaminophen effects. Figure 5 also shows that the DCA + TCA + acetaminophen mixture effect was significantly greater than those induced by each of the individual compounds composing that mixture.

The concentration of the AOPPs produced in response to treatment with the individual chemicals and their mixtures are demonstrated in figure 6. While no significant differences in the production of AOPP were observed with DCA and TCA treatments when compared to the control, a significantly greater production was observed with acetaminophen treatment, when compared with the control. Also, while none of the binary mixtures induced significant production of AOPP when compared with the effects produced by the individual compounds composing them, a significantly greater effect on the production of that biomarker was observed with the DCA + TCA + acetaminophen mixture when compared with those of DCA and TCA, but not acetaminophen effect.

Figure 7 shows the effects of treatment with the individual compounds, as well as with their mixtures on NO production. While DCA effect was not significantly different from that of the control, TCA and acetaminophen effects were significantly greater than

that induced by the same control. For the binary mixtures: DCA + TCA mixture effect was not significantly different from those of DCA or TCA, DCA + acetaminophen effect was significantly different from DCA but not acetaminophen effect, and TCA + acetaminophen effect was not significantly different from those of either compound composing it. However, the effect of DCA + TCA + acetaminophen mixture was significantly greater than those produced by each of DCA, TCA or acetaminophen.

For the production of lipid peroxidation, TBARS concentrations were determined in response to each chemical and also in response to their mixtures, and the results are shown in figure 8. DCA, TCA and acetaminophen induced significant TBARS production when compared with the control. As for the binary mixtures: DCA + TCA mixture effect was significantly greater than those produced by either compound, DCA + acetaminophen effect was significantly greater than that of DCA but not of acetaminophen, and TCA + acetaminophen effect was not significantly different from those induced by TCA or acetaminophen. However, DCA+ TCA + acetaminophen mixture effect was significantly greater than those induced by the individual compounds composing it.

Chapter 5

Discussion

Alpha mouse liver (AML- 12) cells were used in this study because previous *in vivo* studies on the effects of DCA and TCA found the compounds to be mainly hepatotoxic/ hepatocarcinogenic in mice after acute and long term exposures (35, 64), and acetaminophen is a known heptotoxicant when administered at high doses to humans and animals (65, 66). In addition, DCA and TCA effects were previously tested in AML 12 cells and were found to induce time and concentration dependent increases in cell death that were assocaited with the induction of OS. Acetaminophen is one of the most commonly and frequently used analgesic drugs. It is therefore important to understand its interaction with DCA and TCA, since the two agents are present in the drinking water that is consumed by humans during their life time. Studying the possible interactions between the three compounds in an *in vivo* system would be time consuming and costly. Therefore, *in vitro* studies on mouse liver cells may provide a rapid and less costly system to screen the effects of different mixtures and to identify the specific mixtures that need to be investigated further in an *in vivo* system. Also, AML-12 cells posses granular cytoplasm, peroxisomes and canalicular like structures, identical to those of the humans (60).

Although previous studies on the effects of DCA and TCA on AML-12 cells found the compounds to induce significant and concentration-dependent decreases in cellular viability, those effects did never reach maximum after 48 hours of incubation (63). Therefore, that time point was used to study acetaminophen effects , and concentrations of the three compounds used for the mixtures were based on their effects at that time point.

To determine whether the mixtures effects would be additive (equal to the sum of the individual effects of the compounds composing the mixtures), less than additive (less than the sum of the effects of the individual compounds composing the mixtures) or more than additive (more than the sum of the effects of the individual compounds composing the mixtures), we tested DCA, TCA and acetaminophen at concentrations that produced 25% of maximal response (cell death), when each was added to the cells. This design would allow observing not only possible additive effects (50% in binary mixtures and 75% in the 3-compound mixture) but also provides possibilities of observing greater than additive effects (> 50% or 75%). The resutls of the study clearly indicated additive effects of the tested mixtures on celluar viability.

Except for the AOPPs production that was only induced significantly by acetaminophen, and NO production that was not significantly induced by DCA, all of the other biomarkers were significantly induced by the three compounds when added individually. However, the mixtures effects on these biomarkers varied significantly among the binary ones, but were all significantly induced in the 3-compound mixture when compared with each of the compounds composing it. The observed variations in the mixtures effects may be contributed to changes in the process of metabolism that may affect the levels and nature of the metabolites formed. In fact, DCA and TCA were found

to undergo metabolism in the livers of mice producing ROS and other free radicals (28), and were also found to induce production of SA, both *in vivo* (59), and *in vitro* in AML-12 cells (63). Acetaminophen was also found to be metabolized *in vivo* and *in vitro* producing free radicals and ROS including SA and H_2O_2 (23, 67, 68).Production of ROS was assessed in this study by SA and NO. However, many other ROS could have been also produced in response to the mixtures While SA was previously suggested to be associated with the production of cellular death by DCA and TCA, both *in vivo* (35, 64) and *in vitro* (63, 69), NO is known to induce two opposite effects on cells, depending on the concentration. Studies have shown that low concentrations of NO could protect macrophage cells from NO-induced apoptosis through cGMP formation (70); but other studies showed that high NO concentration resulted in harmful effects due to the interaction between NO and SA and the generation of reactive nitrogen oxide species (49). Therefore, in the binary mixtures SA production contributed, at least partially to the observed decline in celluar viability, while NO production could have contributed diffrently to cellular viability depending on the level of its production by the different binary mixtures. However, in the 3-compound mixture, productions of the two ROS were observed to be correlated with the decline in cellular viability and may indicate their contribution to the induction of cell death by that mixture. Although we suggested here significant contribution of SA and NO to the observed cell death by the 3-compound mixture, ROS and/ or free radicals other than SA and NO may also have been produced and contributed to the observed decline in cellular viability. As for the mechanisms of the induction of cell death by different ROS, the species are known to attack various cellular components leading to celluar damage and death (71). In this study we determined lipid peroxidation

as a marker of cellular membrane damage, and AOPP production as a marker of protein damage. While lipid peroxidation was in general shown not to correlate well with the productions of SA and NO in the binary mixtures, it correlated very well with their production by the 3-compound mixture. This may indicate significant contribution of the two ROS to the observed production of lipid peroxidation, and ultimate cell death induction by the that mixture. It may also indicate that the two ROS had either a non or partial contribution to the cell death induced by the binary mixtures. This may also confirm our previous suggestion about possibe contribution of free radicals and ROS, other than SA and NO to the production of cell death by all of the mixtures, including the 3-compound one. For example SA and NO can react producing the more damaging peroxynitrate radical that can lead to various cellular damage and is also considered as one pathway for AOPPs formation due to its strong potential to interact with the proteins (72) . Also, SA can be dismutated by SOD and be converted to H_2O_2, which is more damaging ROS than SA (36). In fact, SOD activity was previously found to increase in AML-12 cells in response to different concentrations of DCA and TCA, after 48 h of incubation (63). Since the DCA and TCA concentrations used in this study were within the range of those previously tested, SOD activity was expected to increase leading to increased production of H_2O_2 and to its contribution to the observed cell death. However, future determination of enzyme activity in response to the mixtures is needed to confirm that suggestion.As for the AOPPs production, the biomarker was only significantly induced by acetaminophen and by the 3-compound mixture. Since the level of AOPPs in the 3-compound mixture was not significantly different from that induced by acetaminophen, it is expected that AOPPs production in that mixture was contributed by acetaminophen only. In conclusion, DCA,

TCA and acetaminophen induced significant production of NO and SA, and that these species have contributed significatnly, but not solely to the productions of lipid peroxidation and AOPPs in the 3-compound mixtures. Also, lipid peroxidation and AOPPs contributed significatnly, but not solely to the induction of cellular death by that mixture. However, the contributions of those biomarkers to the observed declines in cellular viabilities in response to the binary mixtures can not be strongly confirmed at this point.

Chapter 6

Future Studies

1. While the results of the study provided evidence for the contribution of the tested biomarkers to the induction of cellular death by the the 3-compound mixture, they did not provide such evidence in regard to the effects of the binary mixtures. Therefore future studies will be mainly focused on mixtures containing the three compounds. This is also more realistic, since DCA and TCA co-exist in the water and human exposure to acetaminophen and water containing the other two compounds resembles the mixture of the three compounds, rather than the binary mixtures.

2. Since it was concluded that the tested biomarkers did not fully contribute to the observed decline in cellular viability by the 3-compound mixture, the roles of other biomarkers of OS may also need to be assessed .

3.Since it was concluded that SOD may paly a role in the induction of the observed effects by the 3-compound mixture, this and other antioxidant enzyme activities may need to be determined and correlated with the observed effects.

4.Glutahione is one of the most important antioxidants in the biological system that is known to be depleted in response to high doses of acetamiophen. Therefore, the role of this

biomarker in the 3-compound mixture-induced decline in cellular vaibility needs to be assessed.

5.Since it was suggested that the process of metabolism of the three compounds may be altered when the compounds exist in mixtures, it is important to study the nature and levels of metabolites formed in a 3-compound mixture system and compare that with those formed in systems containing the individual compounds. It is also important to study the 3-compound mixture effects in an *in vivo* system to determine not only the role of metabolism, but also the possible contribution of the other toxicokinetic processes, including absorption, distribution and excretion to the net effect of the mixture.

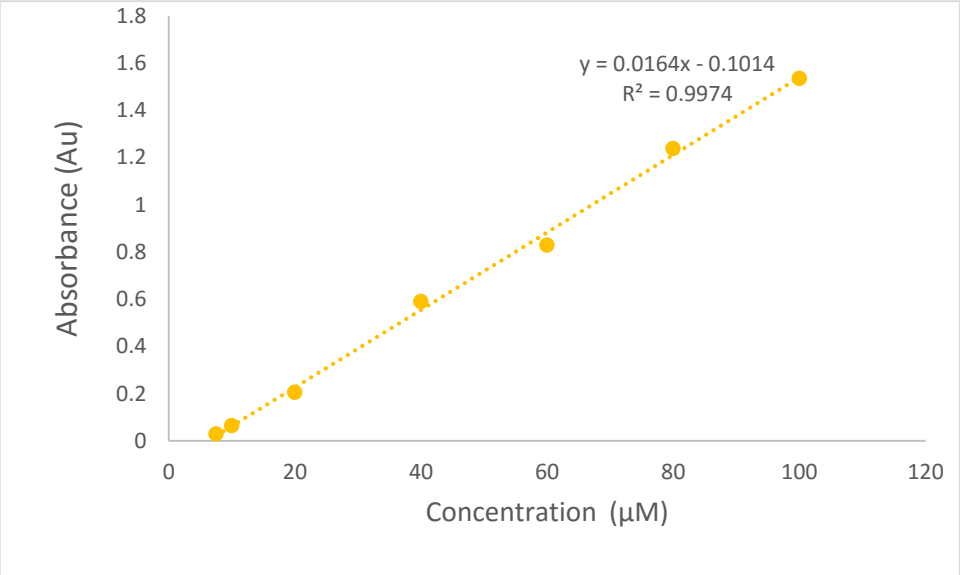

Figure 1. Standard curve of AOPPs, using chloramine T solution

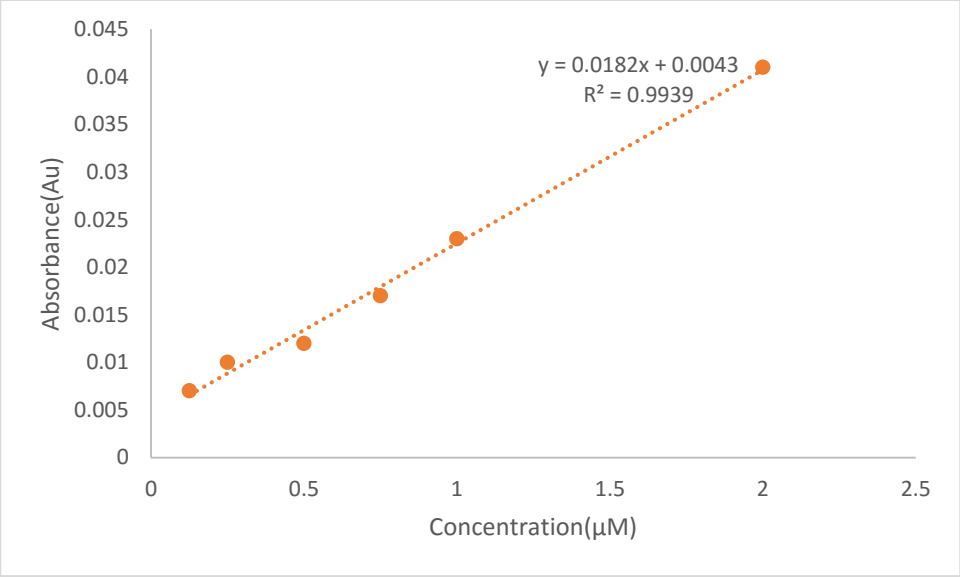

Figure 2. Standard curve of nitric oxide, using sodium nitrite

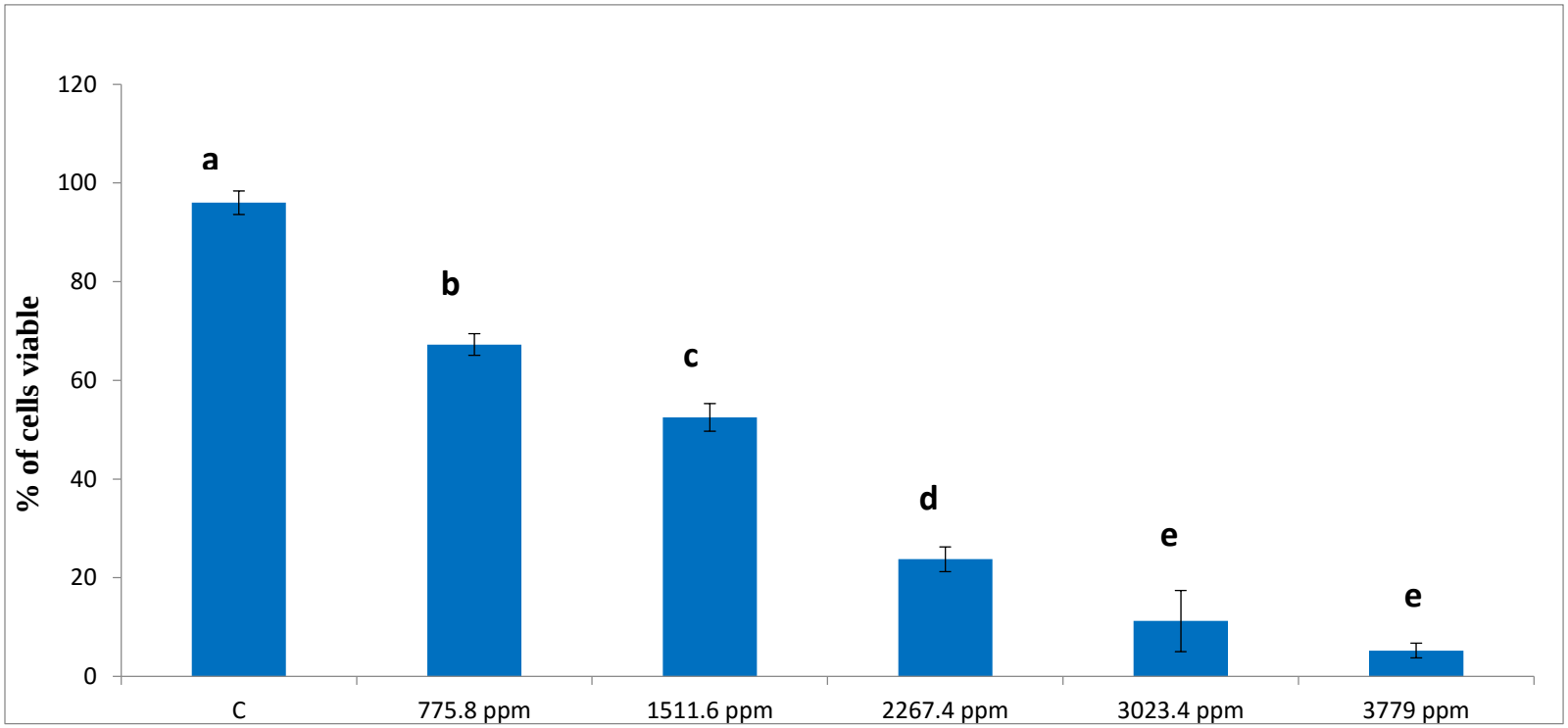

Figure 3 Cellular viabilities in response to treatment with different concentrations of acetaminophen. Columns with non- identical superscripts are significantly different, $p < 0.05$

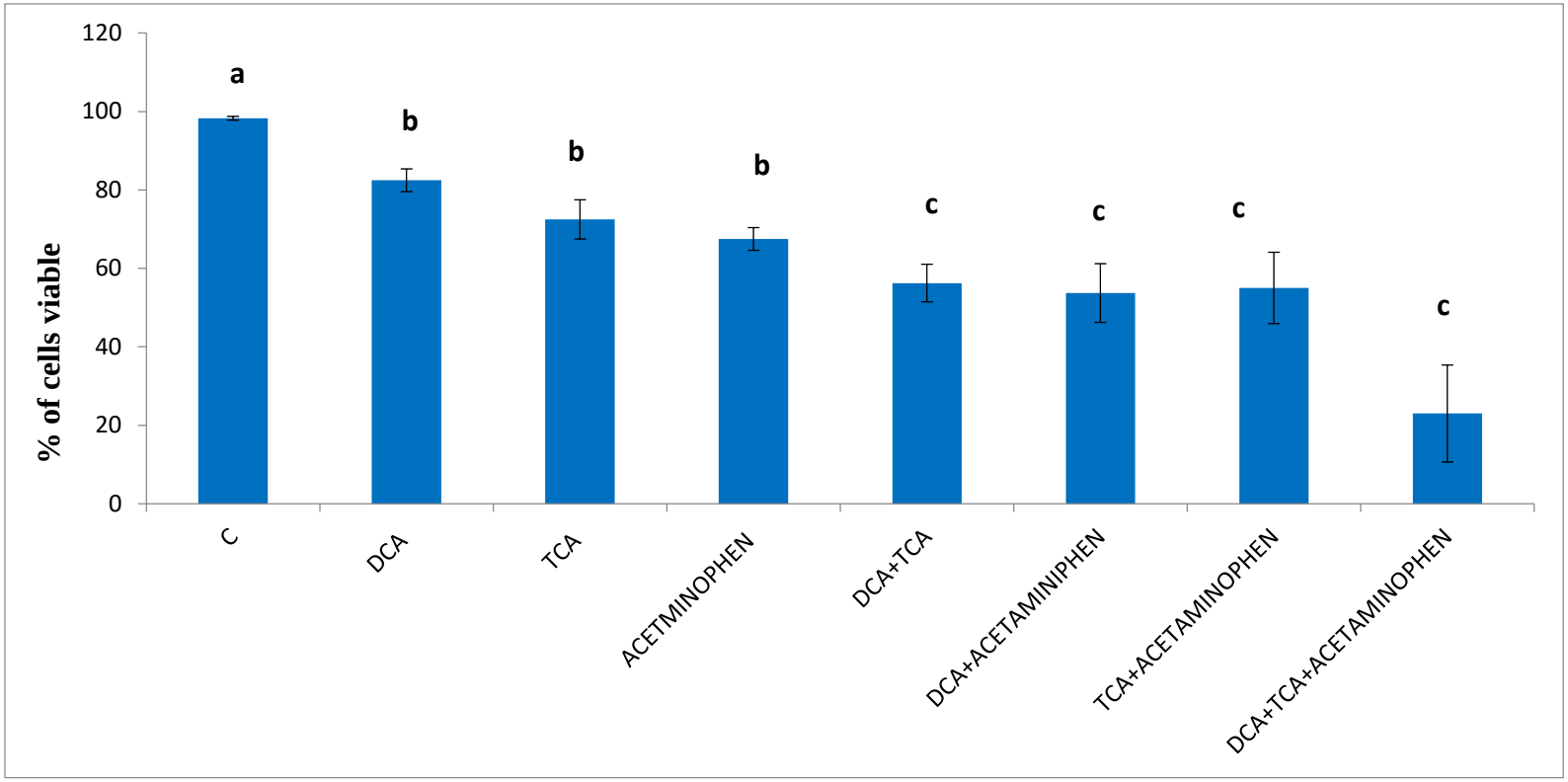

Figure 4. Cellular viability in response to treatment with DCA, TCA, acetaminophen and mixtures of the compounds for 48 hours. Individual compounds were compared with the control, and mixtures were compared with the individual compounds composing them. Columns with non-identical superscripts are significantly different, $p < 0.05$

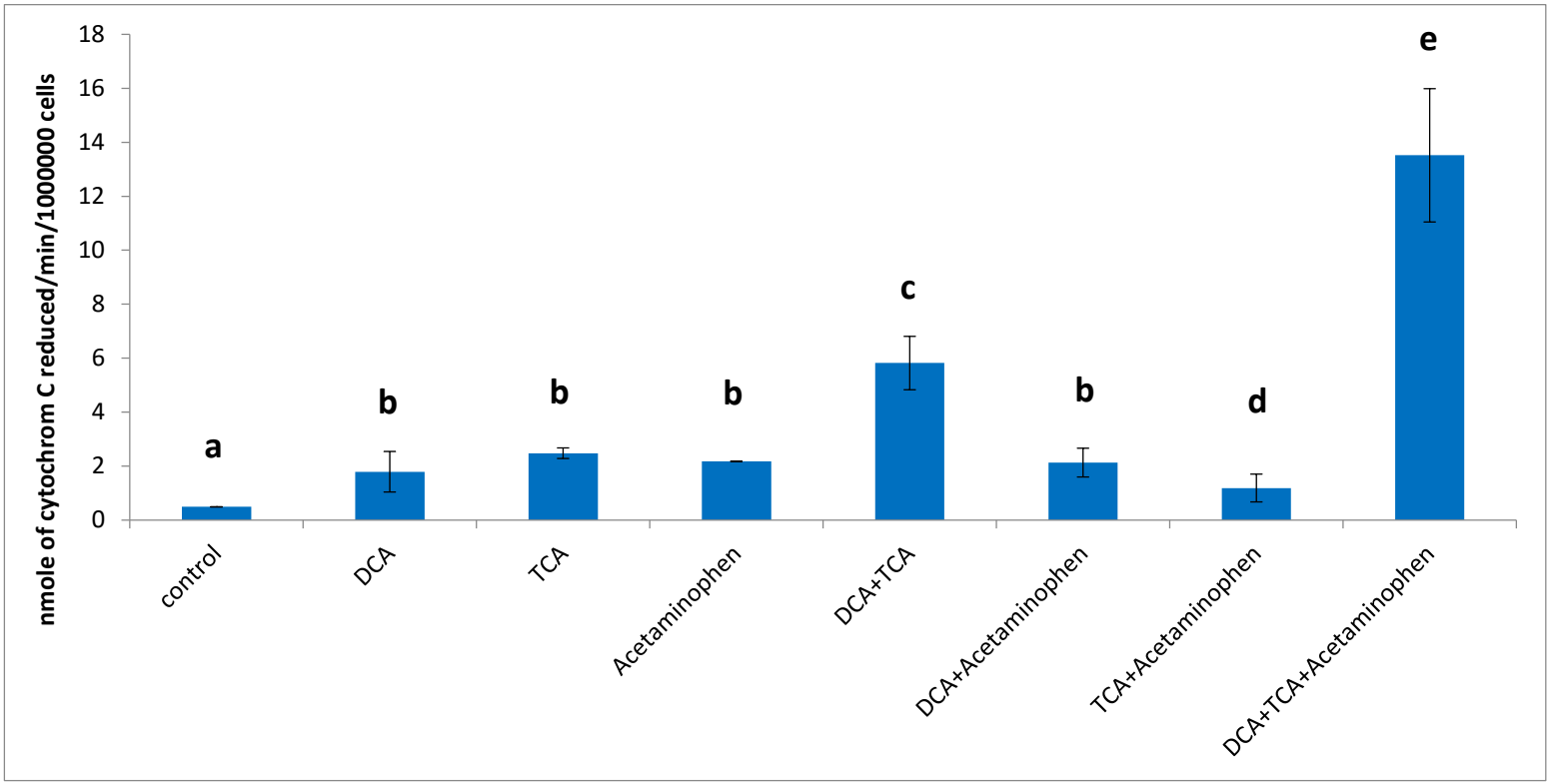

Figure 5. SA production measured as cytochrome c reduced / min/ 10^{6} cells in response to cellular treatment with DCA, TCA, acetaminophen and mixtures of the compounds for 48 hours. The individual compounds effects were compared with that of the control and mixtures effects were compared with those of the individual compounds composing them. Columns with non-identical superscripts are significantly different, $p>0.05$

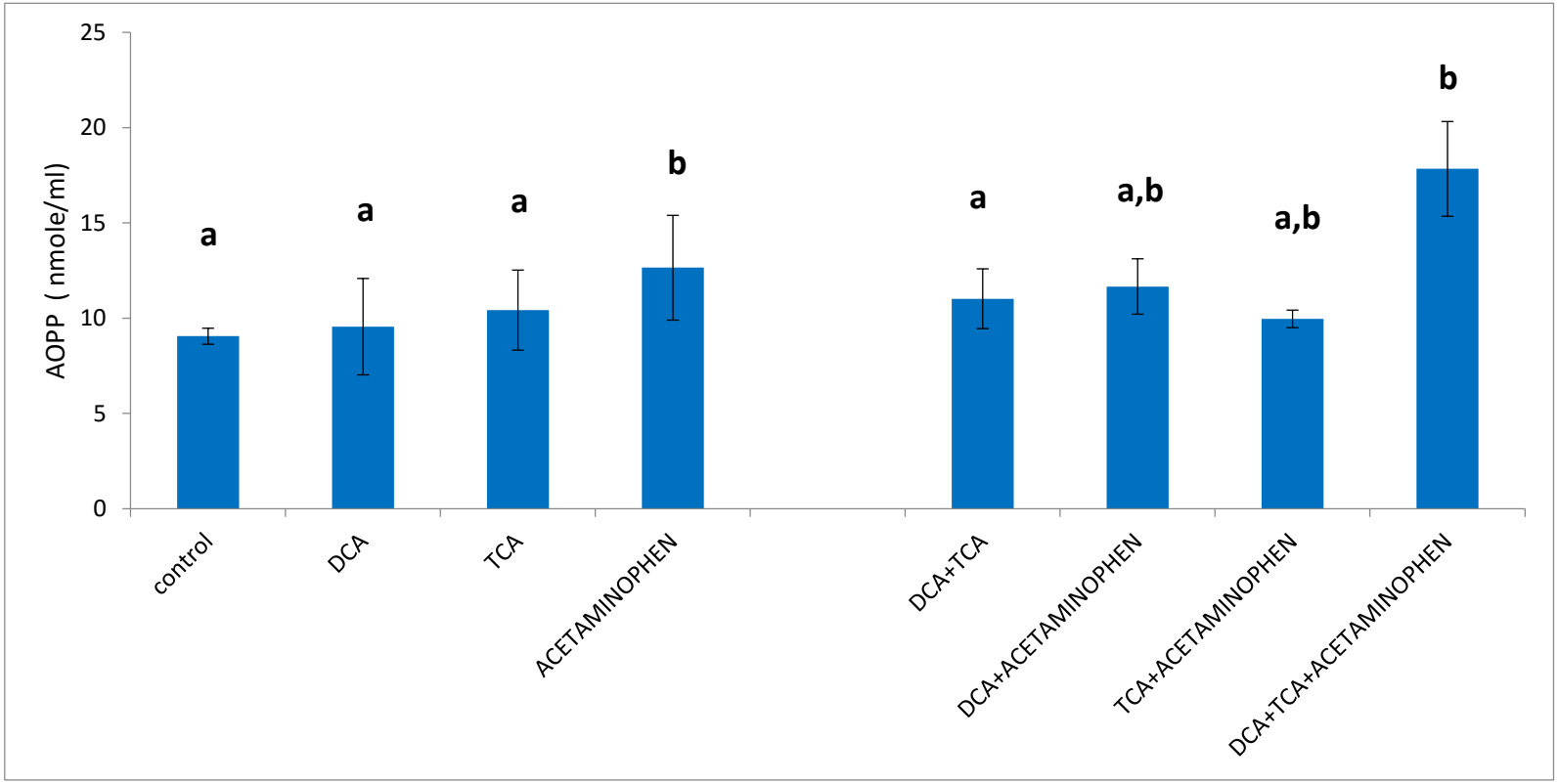

Figure 6. AOPP production in response to cellular treatment with DCA, TCA, acetaminophen and mixtures of the compounds after incubation for 48 hours. The individual compounds effects were compared with the control, and mixtures effects were compared with the individual compounds composing them. Columns sharing an identical superscript are not significantly different, $p > 0.05$

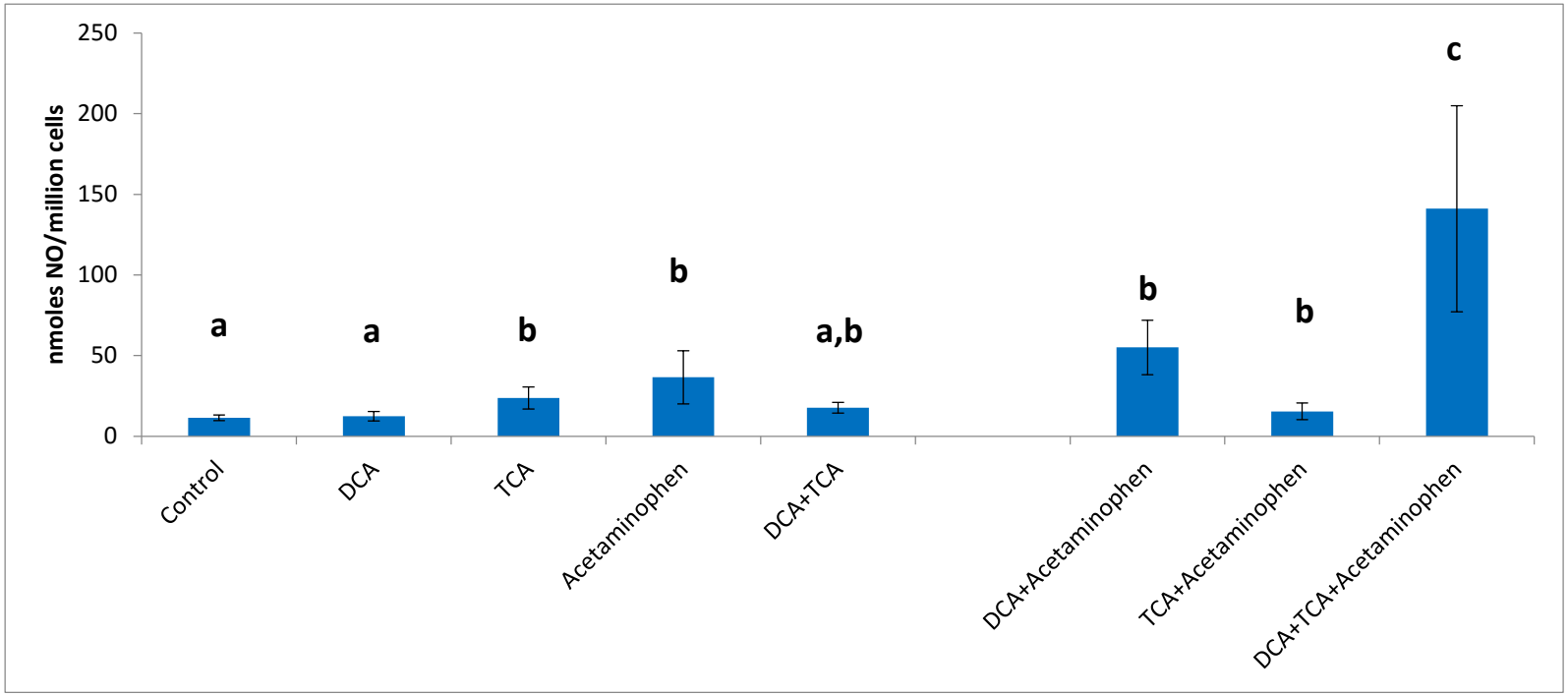

Figure 7. NO production in response to cellular treatment with DCA, TCA, acetaminophen, and mixtures of the compounds after incubation for 48 hours. Each individual compounds effect was compared with the control, and mixtures effects were compared with those of the individual compounds composing them. Columns sharing an identical superscript are not significantly different, $p > 0.05$

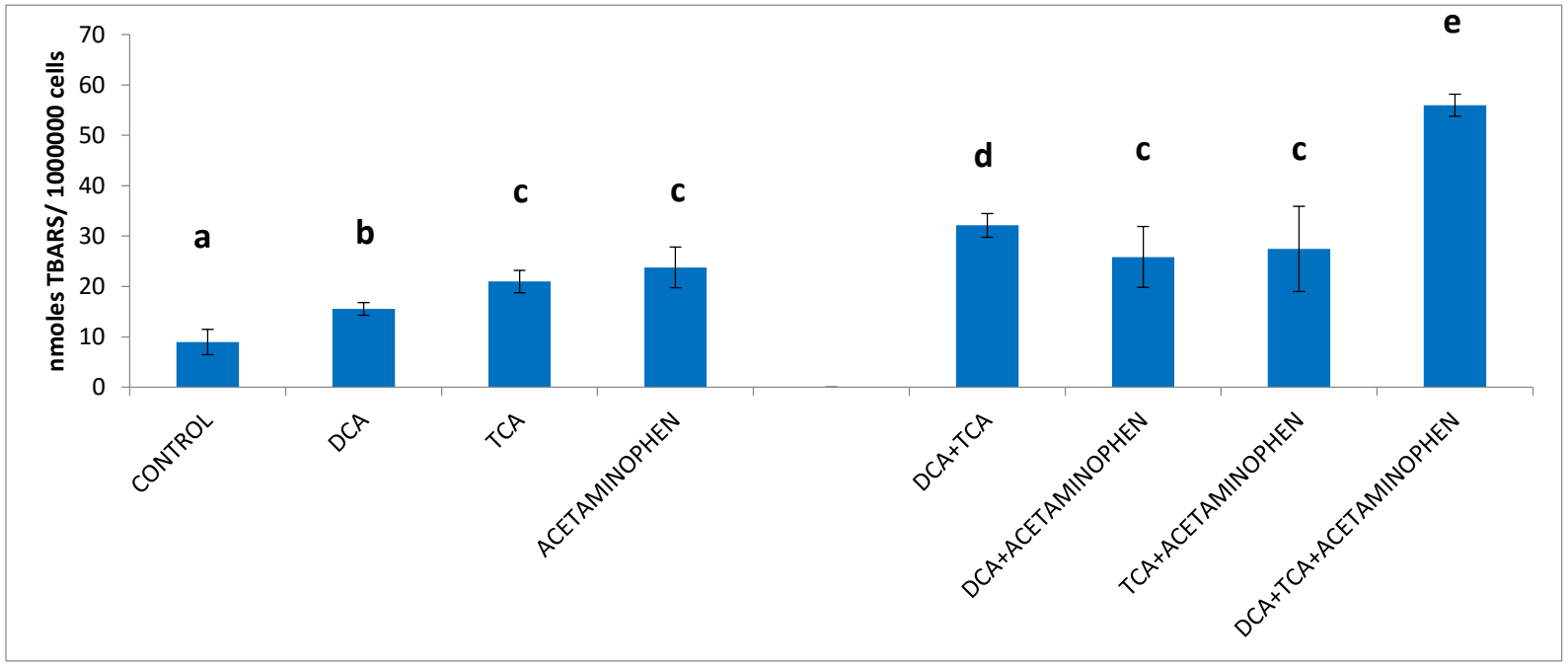

Figure 8. TBARS production in response to cellular treatment with DCA, TCA, acetaminophen and mixtures of the compounds for 48 hours. Each individual compounds effect was compared with the control and mixtures effects were compared with those of the individual compounds composing them. Columns with non-identical superscript are significantly different, p ,0.05

References

1. Water quality and health council 2003. Available from: http://www.waterandhealth.org/drinkingwater/wp.html.

2. Center for disease control and prevention 2015. Available from: http://www.cdc.gov/healthywater/drinking/public/chlorine-disinfection.html.

3. Center for disease control and prevention 2014. Available from: http://www.cdc.gov/safewater/chlorination-byproducts.html.

4. Nikolaou AD, Golfinopoulos SK, Lekkas TD, Kostopoulou MN. DBP levels in chlorinated drinking water: effect of humic substances. Environmental monitoring and assessment. 2004;93(1-3):301-19.

5. Ketcha M, Stevens D, Warren D, Bishop C, Brashear W. Conversion of trichloroacetic acid to dichloroacetic acid in biological samples. Journal of analytical toxicology. 1996;20(4):236-41.

6. Sies H. What is oxidative stress? Oxidative Stress and Vascular Disease: Springer; 2000. p. 1-8.

7. Yoshikawa T, Naito Y. What is oxidative stress? Japan Medical Association Journal. 2002;45(7):271-6.

8. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology. 2007;39(1):44-84.

9. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy reviews. 2010;4(8):118.

10. Lee J, Koo N, Min D. Reactive oxygen species, aging, and antioxidative nutraceuticals. Comprehensive reviews in food science and food safety. 2004;3(1):21-33.

11. Benzi G, Moretti A. Are reactive oxygen species involved in Alzheimer's disease? Neurobiology of aging. 1995;16(4):661-74.

12. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circulation research. 2000;87(10):840-4.

13. Tao F, Gonzalez-Flecha B, Kobzik L. Reactive oxygen species in pulmonary inflammation by ambient particulates. Free Radical Biology and Medicine. 2003;35(4):327-40.

14. Busciglio J, Yankner BA. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. Nature. 1995;378(6559):776-9.

15. Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. Oxidative stress and cell signalling. Current medicinal chemistry. 2004;11(9):1163-82.

16. Björkman R, Hallman K, Hedner J, Hedner T, Henning M. Acetaminophen blocks spinal hyperalgesia induced by NMDA and substance P. Pain. 1994;57(3):259-64.

17. National Cancer Institue 2015. Available from: https://ncit.nci.nih.gov/ncitbrowser/ConceptReport.jsp?dictionary=NCI_Thesaurus&ns=NCI_Thesaurus&code=C198.

18. Chandrasekharan N, Dai H, Roos KLT, Evanson NK, Tomsik J, Elton TS, et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proceedings of the National Academy of Sciences. 2002;99(21):13926-31.

19. Vijayakaran K, Kannan K, Kesavan M, Suresh S, Sankar P, Tandan SK, et al. Arsenic reduces the antipyretic activity of paracetamol in rats: modulation of brain COX-2 activity and CB 1 receptor expression. Environmental toxicology and pharmacology. 2014;37(1):438-47.

20. Granberg RA, Rasmuson ÅC. Solubility of paracetamol in pure solvents. Journal of Chemical & Engineering Data. 1999;44(6):1391-5.

21. James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. Drug metabolism and disposition. 2003;31(12):1499-506.

22. McGill MR, Yan HM, Ramachandran A, Murray GJ, Rollins DE, Jaeschke H. HepaRG cells: a human model to study mechanisms of acetaminophen hepatotoxicity. Hepatology. 2011;53(3):974-82.

23. McGill MR, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. The Journal of clinical investigation. 2012;122(4):1574-83.

24. Gonzalez-Leon A, Schultz IR, Xu G, Bull RJ. Pharmacokinetics and metabolism of dichloroacetate in the F344 rat after prior administration in drinking water. Toxicology and applied pharmacology. 1997;146(2):189-95.

25. Singer PC, Obolensky A, Greiner A. DBPs in chlorinated North Carolina drinking waters. Journal-American Water Works Association. 1995;87(10):83-92.

26. Xu G, Stevens DK, Bull RJ. Metabolism of bromodichloroacetate in B6C3F1 mice. Drug metabolism and disposition. 1995;23(12):1412-6.

27. Abbas R, Fisher JW. A physiologically based pharmacokinetic model for trichloroethylene and its metabolites, chloral hydrate, trichloroacetate, dichloroacetate, trichloroethanol, and trichloroethanol glucuronide in B6C3F1 mice. Toxicology and applied pharmacology. 1997;147(1):15-30.

28. Larson J, Bull R. Metabolism and lipoperoxidative activity of trichloroacetate and dichloroacetate in rats and mice. Toxicology and applied pharmacology. 1992;115(2):268-77.

29. Merdink JL, Gonzalez-Leon A, Bull RJ, Schultz I. The extent of dichloroacetate formation from trichloroethylene, chloral hydrate, trichloroacetate, and trichloroethanol in B6C3F1 mice. Toxicological Sciences. 1998;45(1):33-41.

30. Toxopeus C, Frazier JM. Kinetics of trichloroacetic acid and dichloroacetic acid in the isolated perfused rat liver. Toxicology and applied pharmacology. 1998;152(1):90-8.

31. Lee JB, Chung WG, Kwahck H, Lee KH. Focal treatment of acne scars with trichloroacetic acid: chemical reconstruction of skin scars method. Dermatologic surgery. 2002;28(11):1017-21.

32. Herren-Freund SL, Pereira MA, Khoury MD, Olson G. The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver. Toxicology and applied pharmacology. 1987;90(2):183-9.

33. DeAngelo AB, Daniel FB, Stober JA, Olson GR. The carcinogenicity of dichloroacetic acid in the male B6C3F1 mouse. Toxicological Sciences. 1991;16(2):337-47.

34. Pereira MA. Carcinogenic activity of dichloroacetic acid and trichloroacetic acid in the liver of female B6C3F1 mice. Toxicological Sciences. 1996;31(2):192-9.

35. Hassoun EA, Dey S. Dichloroacetate-and trichloroacetate-induced phagocytic activation and production of oxidative stress in the hepatic tissues of mice after acute exposure. Journal of biochemical and molecular toxicology. 2008;22(1):27-34.

36. Dröge W. Free radicals in the physiological control of cell function. Physiological reviews. 2002;82(1):47-95.

37. Dringen R, Hamprecht B. Involvement of glutathione peroxidase and catalase in the disposal of exogenous hydrogen peroxide by cultured astroglial cells. Brain research. 1997;759(1):67-75.

38. Barbusiński K. Fenton reaction-controversy concerning the chemistry. Ecological Chemistry and Engineering S. 2009;16(3):347-58.

39. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney international. 1996;49(5):1304-13.

40. Piwowar A. [Advanced oxidation protein products. Part I. Mechanism of the formation, characteristics and property]. Polski merkuriusz lekarski: organ Polskiego Towarzystwa Lekarskiego. 2010;28(164):166-9.

41. Marsche G, Frank S, Hrzenjak A, Holzer M, Dirnberger S, Wadsack C, et al. Plasma-advanced oxidation protein products are potent high-density lipoprotein receptor antagonists in vivo. Circulation research. 2009;104(6):750-7.

42. Avinash S, Anitha M, Chandran V, Rao GM, Sudha K, Shetty BV. Advanced oxidation protein products and total antioxidant activity in colorectal carcinoma. 2009.

43. Baskol G, Demir H, Baskol M, Kilic E, Ates F, Karakukcu C, et al. Investigation of protein oxidation and lipid peroxidation in patients with rheumatoid arthritis. Cell biochemistry and function. 2006;24(4):307-12.

44. Witko-Sarsat V, Nguyen-Khoa T, Jungers P, Drüeke T, Descamps-Latscha B. Advanced oxidation protein products as a novel molecular basis of oxidative stress in uraemia. Nephrology Dialysis Transplantation. 1999;14(suppl 1):76-8.

45. Ali EM, Soha H, Mohamed TM. Nitric oxide synthase and oxidative stress: regulation of nitric oxide synthase. Oxidative stress—molecular mechanisms and biological effects InTech. 2012:61-72.

46. Luiking YC, Engelen MP, Deutz NE. Regulation of nitric oxide production in health and disease. Current opinion in clinical nutrition and metabolic care. 2010;13(1):97.

47. Besco R, Sureda A, Tur JA, Pons A. The effect of nitric-oxide-related supplements on human performance. Sports medicine. 2012;42(2):99-117.

48. Rosselli M, Keller R, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Human Reproduction Update. 1998;4(1):3-24.

49. Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radical Biology and Medicine. 1998;25(4):434-56.

50. Mylonas C, Kouretas D. Lipid peroxidation and tissue damage. In vivo (Athens, Greece). 1998;13(3):295-309.

51. Ramana KV, Srivastava S, Singhal SS. Lipid peroxidation products in human health and disease. Oxidative medicine and cellular longevity. 2013;2013.

52. Gibson JD, Pumford NR, Samokyszyn VM, Hinson JA. Mechanism of acetaminophen-induced hepatotoxicity: covalent binding versus oxidative stress. Chemical research in toxicology. 1996;9(3):580-5.

53. Reid AB, Kurten RC, McCullough SS, Brock RW, Hinson JA. Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and mitochondrial permeability transition in freshly isolated mouse hepatocytes. Journal of Pharmacology and Experimental Therapeutics. 2005;312(2):509-16.

54. Hinson JA, Reid AB, McCullough SS, James LP. Acetaminophen-induced hepatotoxicity: role of metabolic activation, reactive oxygen/nitrogen species, and mitochondrial permeability transition. Drug metabolism reviews. 2004;36(3-4):805-22.

55. Adamson GM, Harman AW. Oxidative stress in cultured hepatocytes exposed to acetaminophen. Biochemical pharmacology. 1993;45(11):2289-94.

56. Arnaiz SL, Llesuy S, Cutrín JC, Boveris A. Oxidative stress by acute acetaminophen administration in mouse liver. Free Radical Biology and Medicine. 1995;19(3):303-10.

57. Hassoun EA, Cearfoss J. Dichloroacetate-and trichloroacetate-induced modulation of superoxide dismutase, catalase, and glutathione peroxidase activities and glutathione level in the livers of mice after subacute and subchronic exposures. Toxicological & Environmental Chemistry. 2011;93(2):332-44.

58. Austin EW, Parrish JM, Kinder DH, Bull RJ. Lipid peroxidation and formation of 8-hydroxydeoxyguanosine from acute doses of halogenated acetic acids. Toxicological Sciences. 1996;31(1):77-82.

59. Hassoun E, Cearfoss J, Mamada S, Al-Hassan N, Brown M, Heimberger K, et al. The effects of mixtures of dichloroacetate and trichloroacetate on induction of oxidative

stress in livers of mice after subchronic exposure. Journal of Toxicology and Environmental Health, Part A. 2014;77(6):313-23.

60. Wu JC, Merlino G, Fausto N. Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor alpha. Proceedings of the National Academy of Sciences. 1994;91(2):674-8.

61. Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. Journal of Chromatography B. 2007;851(1):51-70.

62. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical biochemistry. 1978;86(1):271-8.

63. Hassoun E, Mettling C. Dichloroacetate and Trichloroacetate Toxicity in AML12 Cells: Role of Oxidative Stress. Journal of biochemical and molecular toxicology. 2015;29(11):508-12.

64. Hassoun EA, Cearfoss J, Spildener J. Dichloroacetate-and trichloroacetate-induced oxidative stress in the hepatic tissues of mice after long-term exposure. Journal of Applied Toxicology. 2010;30(5):450-6.

65. Blazka ME, Wilmer JL, Holladay SD, Wilson RE, Luster MI. Role of proinflammatory cytokines in acetaminophen hepatotoxicity. Toxicology and applied pharmacology. 1995;133(1):43-52.

66. Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug metabolism reviews. 2012;44(1):88-106.

67. Hinson JA, Reid AB, McCullough SS, James LP. Acetaminophen-Induced Hepatotoxicity: Role of Metabolic Activation, Reactive Oxygen/Nitrogen Species, and Mitochondrial Permeability Transition. Drug metabolism reviews. 2004;36(3-4):805-22.

68. Groothuis G, Mafirakureva N, Proost J, Jetten M, Kleinjans J, Lommen A, et al., editors. Risk assessment of paracetamol induced liver toxicity based on human in vitro data. The Toxicologist; 2014.

69. Hassoun EA, Ray S. The induction of oxidative stress and cellular death by the drinking water disinfection by-products, dichloroacetate and trichloroacetate in J774. A1 cells. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 2003;135(2):119-28.

70. Yoshioka Y, Yamamuro A, Maeda S. Nitric oxide at a low concentration protects murine macrophage RAW264 cells against nitric oxide-induced death via cGMP signaling pathway. British journal of pharmacology. 2003;139(1):28-34.

71. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. Gene. 2002;286(1):135-41.

72. Servettaz A, Guilpain P, Goulvestre C, Chéreau C, Hercend C, Nicco C, et al. Radical oxygen species production induced by advanced oxidation protein products predicts clinical evolution and response to treatment in systemic sclerosis. Annals of the rheumatic diseases. 2007;66(9):1202-9.