The Role of Iron Chelators in Reducing Lipid Peroxidation

By

Amanda N. Nicholson

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Approved by

Xu (Shine) Zhang, PhD, Verschuren Centre, Cape Breton University

Ken Oakes, PhD, Department of Biology Cape Breton University

Rod Beresford, PhD, Department of Biology, Cape Breton University

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Within biological systems, iron is tightly controlled by transferrin and ferritin, leaving only small amounts of chelatable and redox-active iron in the labile iron pool. Iron can participate in both critical (*i.e*. hemoglobin formation) and dangerous (*i.e*. Reactive Oxygen Species (ROS) generation) processes. When there is an increase in the labile iron pool, iron can readily catalyze Fenton Reactions, resulting in the formation of reactive oxygen species which can lead to oxidative stress. In the Fenton Reaction, Fe^{2+} is oxidized by hydrogen peroxide (H_2O_2) from the mitochondria, generating hydroxide ions and hydroxyl radicals, which are potent, non-selective oxidizing agents that react with biomolecules, such as DNA, amino acids, proteins and lipids. Lipid peroxidation is a chain reaction, initiated by the Fenton Reaction, that can eventually lead to cell death. Excess labile iron can result from various diseases, such as hemochromatosis and thalassemia. Chelators can be used in the treatment of these diseases because they remove iron from solution, thus preventing the Fenton Reaction, and consequently lipid peroxidation. Using an arachidonic acid model, this polyunsaturated fatty acid was exposed to both Fe^{3+} and Fenton Reaction conditions (*i.e*., Fe^{3+} and H_2O_2), to induce lipid peroxidation. The arachidonic acid model was also exposed to iron chelators (*i.e*. DIBI and deferiprone) to assess their ability to supress lipid peroxidation. Malondialdehyde (MDA), a stable by-product of lipid peroxidation, was used to quantify oxidative stress using a thiobarbituric acid reactive substance (TBARS) assay. In the TBARS assay, thiobarbituric acid (TBA) reacts with MDA to form a TBA-MDA chromogen. However, in the arachidonic acid model, increasing concentrations of hydrogen peroxide reduced the fluorescence signal from the TBA-MDA chromogen. The Fenton Reaction interferes with the TBARS assay because the colorimetric dye appears to be degraded by the presence of hydrogen peroxide. Fe^{3+} was sufficient to induce lipid peroxidation in arachidonic

acid and was subsequently used in all experiments. Iron chelators, *i.e.*, deferiprone and DIBI, a polymeric iron chelator, were used to inhibit lipid peroxidation. The multidentate chelator DIBI was more effective at reducing Fe^{3+} induced lipid peroxidation in an *in vitro* arachidonic acid model.

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To the other biology Honours students, it's been a pleasure going through this journey with you. It was not easy pursuing an Honours project, but you've inspired me to work hard and challenge myself. I would also like to thank Chelation Partners and Cape Breton University's Chemistry Department for donating their products and materials for my project.

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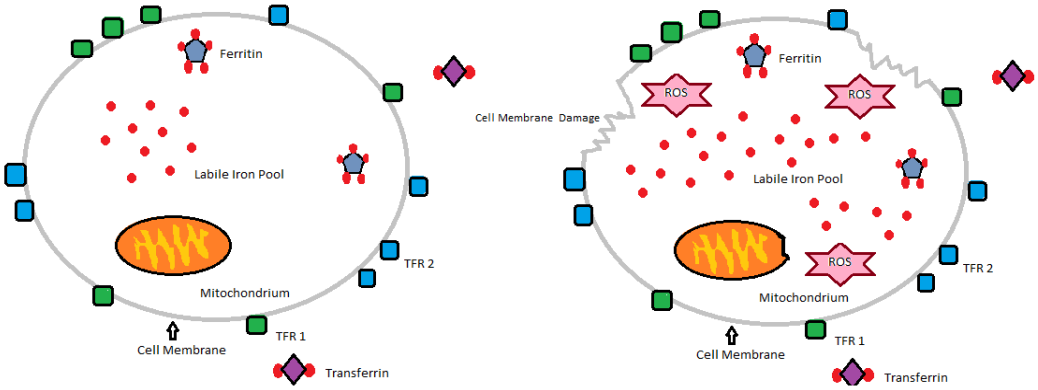
1 Introduction

1.1 Iron and Labile Iron Pool

Iron is naturally found in animal tissues in low concentrations, primarily in hemoglobin and transport proteins. It is highly regulated through a series of controlled mechanisms where iron is transported by transferrin in the plasma and stored by ferritin in the cytoplasm (Galey, 1996). Although iron is necessary for various biological processes, when iron is unregulated, it can catalyse various adverse reactions (Dixon and Stockwell, 2014).

The labile iron pool (LIP) is a pool of both chelatable and redox-active iron ($Fe^{2+}$and Fe^{3+}) found within the cytoplasm. The LIP is essential for iron metabolism because it temporarily stores iron brought in the cell before it is stored by ferritin (Cabantchik, 2014). Since there is no passive excretory mechanism for iron, various hereditary factors, diseases and repeated transfusions can cause iron overload (Wood, 2014), which is the accumulation of iron within the LIP (Chutvanichkul *et al*., 2018). Hemochromatosis, for example, is a genetic disorder which causes iron overload (Wood, 2014). The increased iron in the LIP could participate in Fenton Reactions/Haber-Weiss reactions (Kruszewski, 2003).



Normal Iron *Iron Overload*

Figure 1. Schematic representation comparing a cell with normal iron levels and a cell with iron overload. In both cells, iron is transported and stored by transferrin and ferritin, respectively. The cell with normal iron levels generates small concentration of reactive oxygen species (ROS). In comparison, excessive iron in the iron overload cell generates high concentrations of ROS, which cause significant cell damage.

1.2 Fenton Reaction

Hydrogen peroxide is naturally found in the body as products of the respiration cycle or used by macrophages to destroy pathogens. Typically, hydrogen peroxide is removed by catalase, however, if not immediately broken down, it can react with free iron from LIP to produce the Fenton Reaction (Lawson *et al*., 2016). The Fenton Reaction is a redox cycle where both Fe^{2+}or Fe^{3+} react with hydrogen peroxide to generate hydroxyl anions and a hydroxyl radical (Eq.1). Redox cycles continue indefinitely until an antioxidant or a second free radical is encountered (Halliwell and Chirico, 1993). Fe^{3+}can be regenerated to Fe^{2+} by reacting with superoxide radical via Haber-Weiss reaction (Eq. 2; Lawson *et al*., 2016). Hydroxyl radicals are extremely aggressive oxidants and fast reacting molecules which will damage the closest

molecules they encounter. (Lawson *et al*, 2016). These hydroxyl radicals can react with lipids and initiate lipid peroxidation (Gutteridge, 1995).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^+ + OH_2^\bullet$$

Equation 1. The Fenton Reaction is a redox reaction between both Fe^{2+} and Fe^{3+} and hydrogen peroxide which generates hydroxyl radicals and hydroxyl anions.

$$Fe^{3+} + O_2^{-\bullet} \leftrightarrow Fe^{2+} + O_2$$

Equation 2. The Haber-Weiss reaction where Fe^{3+} reacts with superoxide radical to regenerate Fe^{2+} and oxygen which can be reused and continue to generate hydroxyl radicals.

1.3 Lipid Peroxidation

Lipid peroxidation is the oxidative breakdown of polyunsaturated fatty acids (PUFAs) (Nimse and Pal, 2015). There are three stages in the lipid peroxidation chain reaction; (1) initiation, (2) addition, and (3) propagation. Lipid peroxidation is initiated by external oxidants and once this mechanism is initiated, it will continue as a propagation reaction (Minotti and Aust, 1992).

The reaction begins with abstraction of hydrogen from a lipid membrane by a hydroxyl radical, which leave behind an un-paired electron resulting in damage of PUFAs (Repetto *et al,* 2012). PUFAs are a target for lipid peroxidation because of the presence of a double bond, with the methylene bridge being an important target in the initiation of lipid peroxidation. ROS steal an electron from the methylene bridge that results in a water molecule being released and lipid

peroxyl radical being generated. The production of lipid peroxyl radical ultimately results in the production of lipid hydroperoxides, which can abstract hydrogens from other PUFAs, propagating the chain reaction. This propagation reaction continues unabated until two ROS react to form a nonradical; or an antioxidant donates an electron (Halliwell and Chirico, 1993; Repetto *et al,* 2012).

Iron can initiate lipid peroxidation in the absence of hydrogen peroxide, but in its presence, ROS generation is accelerated (Winterbourn, 1995). Fenton's reagents are responsible for initiating lipid peroxidation, requiring either the reduction of Fe^{3+} or oxidation of Fe^{2+}, with some observations of an optimal ratio of Fe^{3+}: Fe^{2+} (Braughler *et al*, 1986). Linoleic and arachidonic acids are PUFAs which are highly susceptible to lipid peroxidation producing a stable product called malondialdehyde (MDA) that can be quantified. As a result, MDA can be used to quantify lipid peroxidation (Yiin and Lin, 1998), which is associated with oxidative stress (Birben *et al*, 2012).

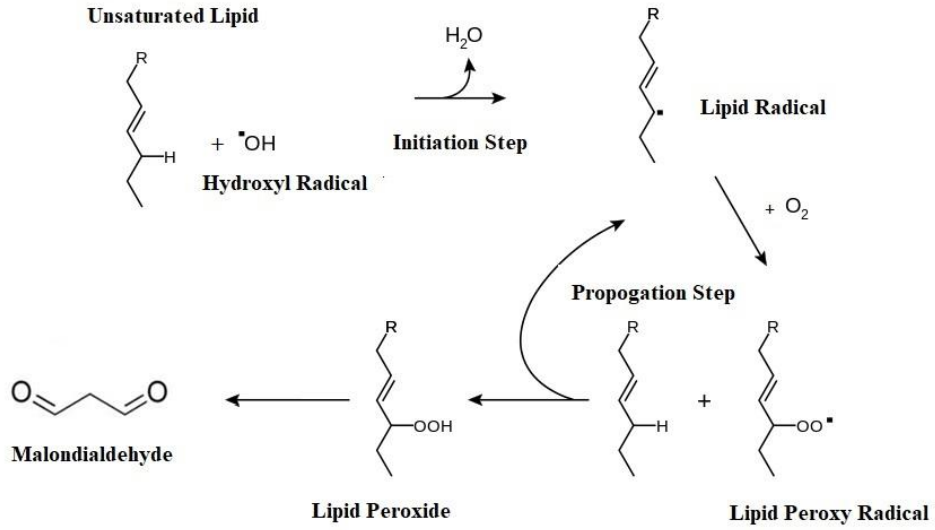


Figure 2. An unsaturated lipid undergoing lipid peroxidation. The hydroxyl radical initiates lipid peroxidation forming lipid radicals, which in turn degrade to form malondialdehyde, a stable product of lipid peroxidation which can be quantified (Modified from Madhura, 2015).

1.4 Reactive Oxygen Species (ROS) and Oxidative Damage

Many ROS are naturally found in cells as by products of cellular processes in mitochondria, chloroplasts and peroxisomes (Apel and Heribert, 2004). ROS can be generated through cellular processes, radiation from the environment, and oxidizing pollutants such as ozone. Oxidative stress can progress to neurological disorders, inflammation, cancer, and ocular and kidney degeneration (Gaschler and Stockwell, 2017). ROS form when oxygen is only partially reduced, resulting in a molecule containing one or more unpaired electrons or free

electron pair (bi-radical), making these molecules extremely unstable and highly reactive (Gutteridge, 1995; Birben *et al*, 2012). ROS can react with another ROS or non-reactive oxygen species. When two ROS react, they form a non-reactive oxygen species. However, when a ROS reacts with a non-reactive oxygen species, a ROS is generated triggering a chain reaction which continuously produces ROS until all ROS react with each other or an antioxidant to form non-reactive oxygen species (Halliwell and Chirico, 1993).

Various antioxidant defense mechanisms are in place to ameliorate ROS production, including dietary antioxidants and inducible enzyme systems. For example, catalase is an antioxidant enzyme that catalyzes the conversion of hydrogen peroxide, to water (Girnun *et al.,* 2002). Vitamin C is a dietary antioxidant that donates a hydrogen to stabilize a ROS which interrupts the ROS chain reaction (Nimse and Pal, 2015). However, under conditions where more ROS are generated than can be removed by the antioxidant defense mechanisms, there is ROS-induced damage of macromolecules (proteins, lipids and nucleic acids) which leads to oxidative damage, tissue damage or cell death (Sachdev, 2008; Apak *et al.*, 2009).

An increase in ROS can stop cell growth or initiate cell death. Cell death occurs through apoptosis, necroptosis or ferroptosis. With apoptosis, ROS cause cell death by blocking essential enzyme pathways. Necroptosis is the non-apoptosis cell death triggered by tumor necrosis factor (TNF) (Dixon and Stockwell, 2014). Finally, ferroptosis is iron dependent cell death which is caused by lethal concentrations of lipid hydroperoxides generated through lipid peroxidation. (Amaral *et al*., 2019).

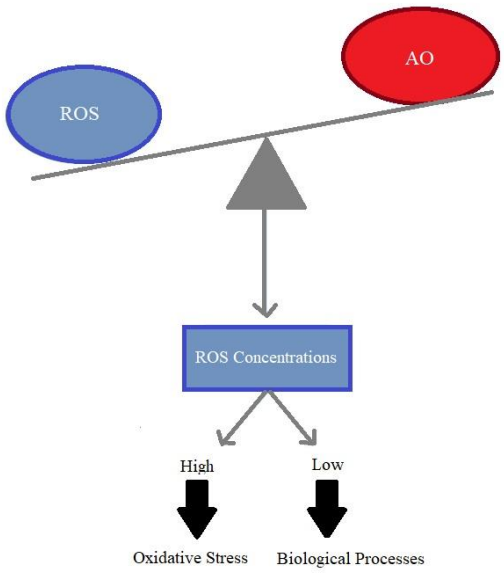


Figure 3. Graphical representation of the balance between reactive oxygen species (ROS) and antioxidants (AO). The increase in the ROS concentration overpowers the antioxidant defence mechanisms, which results in oxidative stress. When AO exceed ROS formed in low concentrations in biological processes, there's little oxidative stress and biological processes continue unabated.

1.5 Proposed Research

1.5.1 Iron Chelators

Iron chelators are commonly used for treating iron overload diseases (Cappellini *et al*., 2014). Chelators form rings structures with metal ions through covalent or co-ordinate bonds, which prevent metals from participating in damaging reactions (Flora and Pachauri, 2010). Iron chelators contain oxygen, nitrogen or sulfur atoms that bind with the low molecular iron within

the LIP (Cappellini *et al*., 2014; Andrew *et al.*, 2016). An ideal iron chelator has a high affinity for Fe^{3+}, low toxicity, and does not interfere with iron homeostasis (Crisponi and Remelli, 2008).

Iron chelators can chelate iron within the labile iron pool and consequently prevent the Fenton Reaction and the initiation of lipid peroxidation (Arora *et al*., 2018). Two promising Fe^{3+} chelators are DIBI and deferiprone (DFP). DIBI, developed by Chelation Partners, is an Fe^{3+} chelator with a hydroxypyridinone-containing iron-chelating polymer that can arrest the growth of *S. aureus*. One DIBI molecule can successfully bind three Fe^{3+} atoms (Ang, 2018). Whereas DFP is a bidentate chelator taken orally by patients who have iron overload. Three DFP molecules are required to bind one Fe^{3+} atom (Olivieri *et al.*, 1995). Previous research suggests that DIBI has a higher avidity for iron and DIBI-bound iron was less available for reactions compared to deferiprone bounds iron (del Carmen Parquet, 2018). Furthermore, the objective of this research is to compare the efficacy of DFP and DIBI chelators in reducing lipid peroxidation.

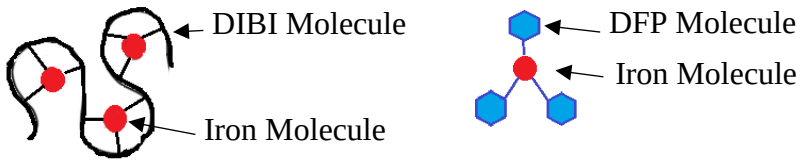


Figure 4. Schematic representation of one DIBI molecule binding three Fe^{3+} molecules. Whereas, three deferiprone (DFP) molecules are needed to bind one Fe^{3+} molecule.

1.5.2 Thiobarbituric Acid Reactive Substances (TBARS) Assay

Thiobarbituric acid reactive-substances (TBARS) assay has been used to estimate lipid peroxidation within lipid membranes and biological systems by quantifying a product of lipid hydroperoxide degradation, malondialdehyde (MDA). MDA is a secondary stable product of lipid peroxidation and has been demonstrated to be useful product to quantality lipid peroxidation (Hodges *et al.,* 1999) as it reacts with two molecules of TBA to produce a red-pink chromogen product (Hodges *et al,* 1999). The TBA-MDA chromogen can be measured photometrically at an absorbance at 532 nm, or a spectrofluorometrically at an excitation/fluorescence of 515/553nm (Meagher, 2000). There are disadvantages of the TBARS assay, particularly non-specific photometric absorbance, yet, this is still a reliable method to estimate lipid peroxidation (Hodges *et al,* 1999). Typically, the TBARS assay is used to quantify MDA within tissue samples, however, various research has used the TBARS assay to quantify lipid peroxidation induced by the Fenton Reaction *in vitro* (Repetto *et al.,* 2010). A novel application of the TBARS assay will be used to quantify the amount of lipid production induced by the Fenton Reaction within an *in vitro* arachidonic acid model.

2 Materials and Methods

2.1 Fatty Acids, Chelating Agents, and Other Chemicals

Arachidonic acid was purchased from VWR International (Mississauga, ON) while linoleic acid was donated by Cape Breton University's Chemistry Department. DIBI chelator was donated by Chelation Partners (Halifax, NS). Deferiprone (DFP) chelator, 1,1,3,3-

tetramethoxypropane (TMP), 20000 poly (ethylene glycol) (PEG), iron chloride (Fe_2Cl_3), ethylenediaminetetraacetic acid (EDTA), 2,6-Di-*tert*-butyl-4-methylphenol, also referred to as butylated hydroxytoluene (BHT), were purchased from Sigma- Aldrich (Oakville, ON). Oleic acid was purchased from Alfa Aesar (Ward Hill, MA). Thiobarbituric acid (TBA) was purchased from MP Biomedical (Solon, OH). Acetone was purchased from Anachemia (Montreal, QC), while 30% hydrogen peroxide (H_2O_2) was purchased from Millpore Corporation (Etobicoke, ON). Acetic acid and 96 well flat black UV plates were purchased from ThermoFisher Scientific (Dartmouth, NS).

2.2 Preparation of Treatments

2.2.1 Treatments Without Chelators

Arachidonic acid was the *in vitro* model chosen to undergo lipid peroxidation/chelator trials, and was exposed to various treatments to generate lipid peroxidation and consequently MDA. Arachidonic acid solution (4mM) was prepared in 0.5% *v/v* acetone. Treatments included 10µM Fe^{3+} only, Fenton Reaction (10µM Fe^{3+} + 20mM H_2O_2), as well as 20mM hydrogen peroxide only. In separate microcentrifuge tubes, individual treatments were added to 200µM arachidonic acid. All microcentrifuge tubes were placed in a 37°C water bath for one hour, followed by the addition of 30µl of EDTA and 30µl of BHT to all microcentrifuge tubes.

2.2.2 Treatments with Chelators

DIBI and DFP chelators were used with Fe^{3+} treatment only and equivalent chelating concentrations of DIBI and DFP (10µM and 30µM, respectively) were employed. The

concentration of Fe^{3+} used with DIBI chelator trials was 30µM, while the concentration of Fe^{3+} used for DFP chelator was 10µM. Before arachidonic acid was added to the treatments, the chelators were exposed to respective iron concentrations for 30 min, enough time for chelators to bind the iron. After 30 min, 7.5µl of arachidonic acid were added to the treatments. Distilled water was added to treatments to bring the total volume to 150µl. Microcentrifuge tubes were placed into a 37°C water bath for one hour prior to 30µl of EDTA and 30µl of BHT being added to arrest any further lipid peroxidation.

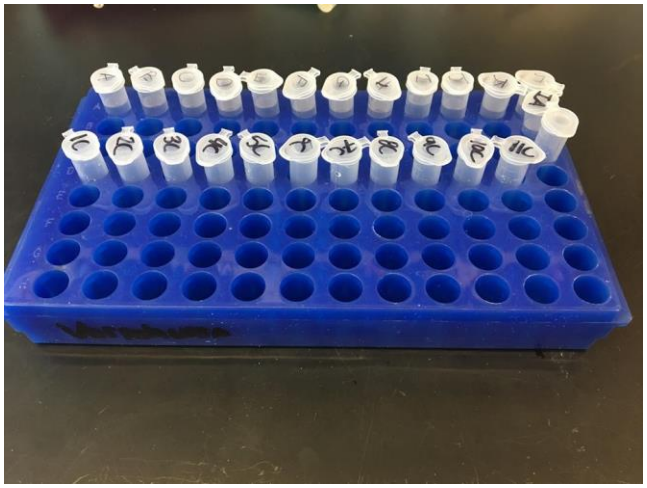


Figure 5. Experimental set up with microcentrifuge tubes containing *in vitro* arachidonic acid model with standards and treatments.

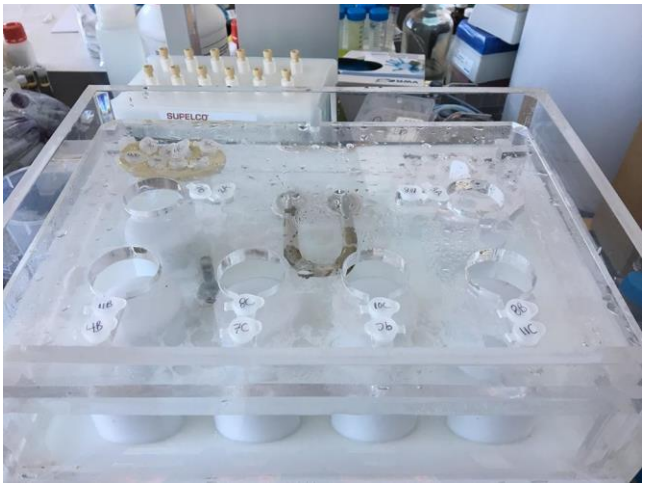


Figure 6. Experimental set up with microcentrifuge tubes containing *in vitro* arachidonic acid model sitting in a 37˚C water bath for one hour. Each microcentrifuge contains a different experimental treatment.

2.3 TBARS Assay Protocol Modified from Oakes and Van Der Kraak (2003)

A standard curve was prepared by hydrolysis of TMP to MDA on an equimolar basis to quantify the concentration of MDA in samples. A 50nmol TMP solution was prepared and subsequently, 13 serial dilutions of TMP were made. Inter assay standards ensured fluorescence intensity and concentration of MDA standards were consistent between experiments and deviated < 10%. To make inter-assay standards, a stock concentration of 0.2nmol TMP was prepared and aliquoted into 20-30 microcentrifuge tubes, each containing 750µl of 0.2nmol TMP solution. Microcentrifuge tubes containing inter assay standards were frozen at -20˚C, and a new aliquot was thawed before each experiment and included in TBARS assay.

The TBARS assay methodology was modified from Oakes and Van Der Kraak (2003), where fluorescence intensity was measured in the aqueous, as opposed to the solvent phase since a tissue extraction was not required. Further, 20,000 PEG was used in place of sodium dodecyl sulfate at a final concentration of 100ppm during TBARS assay. TBARS assay conditions required the addition of, to both standard and sample tubes, 150µl of 20% acetic acid adjusted to pH 3.5, 150µl 0.8% TBA, 61µl of PEG and 10µl of distilled water to each microcentrifuge tube. Both sample and standard containing microcentrifuge tubes were placed hot water bath at 90-100 °C for 30 min. Microcentrifuge tubes were removed from the water bath and allowed to cool to room temperature (20-25°C). Aliquots (100µl) from sampled and standard microcentrifuge tubes were pipetted, in triplicate replicates into Flat Black UV 96-well plate (this prevents the cross-talk experienced by clear plates). Fluorescence intensity was read at 553 nm for emission, following excitation at 515 nm.

Figure 7. Both experimental and standard microcentrifuge tubes during TBARS assay.

Figure 8. Experimental microcentrifuge tubes after completing the TBARS assay.

2.4 TBA-MDA Chromogen Formation

50nmol TMP was made in 50ml distilled H_20 prior to the addition of 750µl of 50nmol TMP to a 2.0ml microcentrifuge tube with 750µl of distilled H_2O, to give a final concentration of 25nmol TMP. TBARS assay was performed as stated in Section 2.3. Subsequently, fluorescence intensity of chromogen was measured, while exposed to Fenton Reaction (Fe^{3+} + H_2O_2), Fe^{3+} and hydrogen peroxide treatments for 30 min.

2.5 Statistical Analysis

All data was assessed for normality using Kolmogorov-Smirnov test and heterogeneity of group variance using Levene's test prior to analysis by ANOVA by using Minitab Version 18.1. All data failed both the non-normal and heterogenous group variance test and was subsequently log transformed; however, data still failed to meet parametric assumptions. Consequently, data was analyzed non-parametrically using Kruskall-Wallis and Mann-Whitney tests. Data were considered significant at $p<0.05$. Fit Regression Model was used to compare the fluorescence intensity equation of TBA-MDA chromogen samples.

3 Results

3.1. Fatty Acids Undergo Lipid Peroxidation

Three polyunsaturated fatty acids (arachidonic acid, linoleic acid, and oleic acid) were exposed to the Fenton Reaction to induce lipid peroxidation and produce concentrations of MDA as a baseline against which the effect of chelators could be measured. Arachidonic acid produced the highest concentration of MDA (0.147nmol/mL), linoleic acid produced intermediate concentrations of MDA (0.126nmol/mL) with oleic acid being the least susceptible to lipid peroxidation with 0.082nmol/mL of MDA produced. MDA concentrations produced by arachidonic acid and linoleic acid did not differ ($p=0.331$). Similarily, nor did MDA produced from linoleic acid and oleic acid ($p=0.251$). Arachidonic acid produced more MDA than oleic acid ($p<0.001$) and was further used as the fatty acid substrate for all subsequent trials.

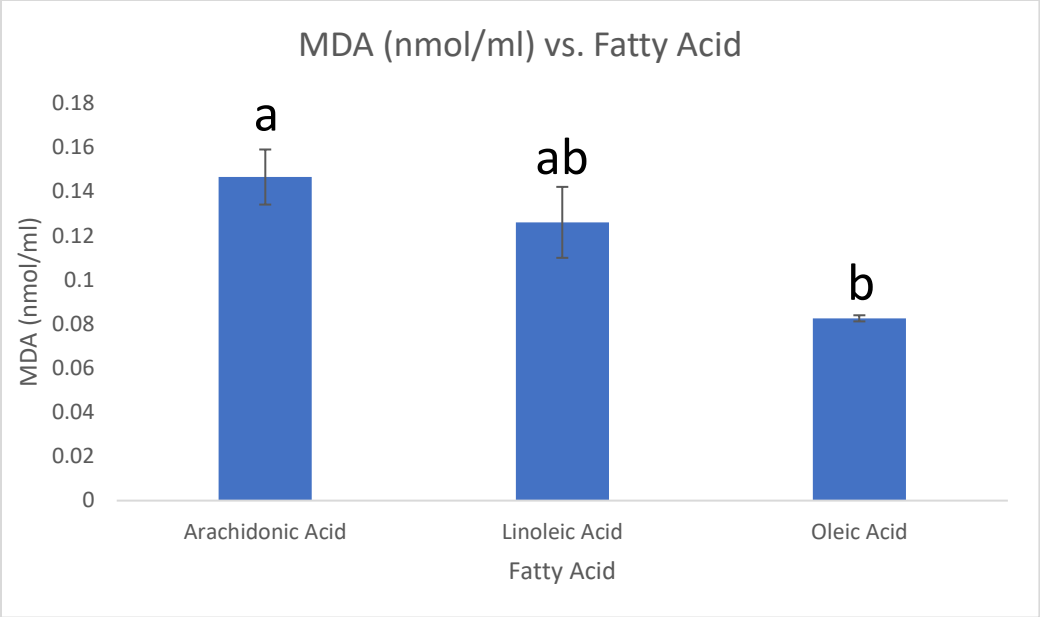


Figure 9. Various fatty acids including arachidonic acid, linoleic acid, and oleic acid were exposed to the Fenton Reaction to induce lipid peroxidation and consequently produce concentration of malondialdehyde. Mann-Whitney statistical analysis test was used, and lower-case letters indicate statistical significance between fatty acids at $\alpha<0.05$. Mean ± SEM and n = 3.

3.2. Comparing MDA Concentrations Induced by $Fe^{3+}$and Fenton Reaction (10µM Fe^{3+} + 20 mM H_2O_2) Treatments

The arachidonic acid *in vitro* model was exposed to 10 µM Fe^{3+} and Fenton Reaction treatment (10 µM Fe^{3+} + 20 mM H_2O_2). Both treatments induced lipid peroxidation and produced concentrations of MDA. However, the Fe^{3+} treatment produced higher concentrations ($p<0.001$) of MDA (0.103 nmol/ml) compared to Fenton Reaction treatment (0.047 nmol/ml).

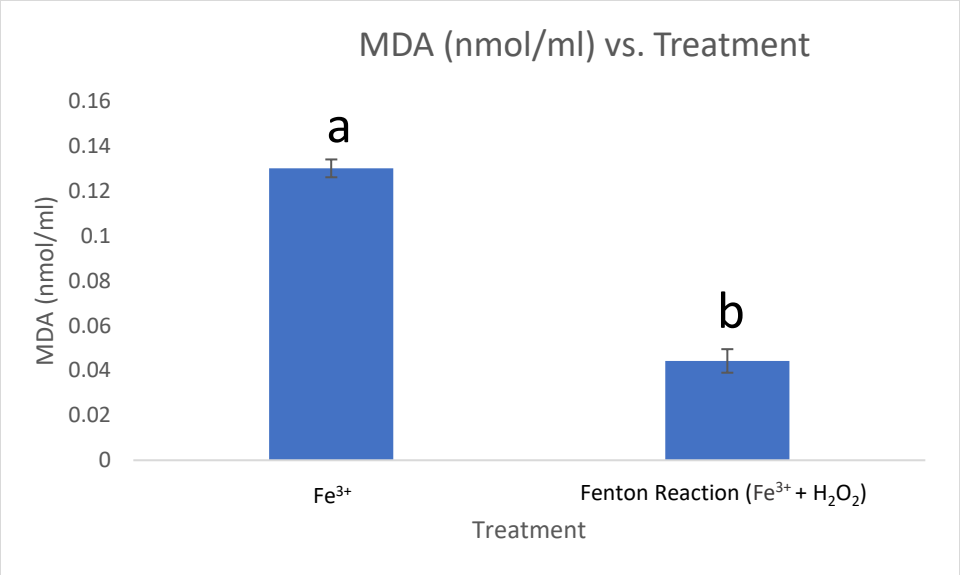


Figure 10. The *in vitro* arachidonic acid model exposed to Fe^{3+} and Fenton Reaction (Fe^{3} + H_2O_2) treatments. These treatments induced lipid peroxidation and produced concentrations of MDA. Mann-Whitney statistical analysis test was used, and lower-case letters indicate statistical significance between treatments at $\alpha<0.05$. Mean ± SEM and $n = 3$.

3.3 Increasing H_2O_2 in Fenton Reaction (Fe^{3+} + H_2O_2) Decreases Concentration of MDA

To optimize the ratio of the Fenton reactants in the production of hydroxyl radicals, the arachidonic acid *in vitro* model was exposed in a series of experiments to fixed iron concentrations with increasing concentrations of hydrogen peroxide. There was a significant decrease in the concentration of MDA produced as the concentration of hydrogen peroxide in the Fenton Reaction treatment increased. Arachidonic acid on its own produced small concentrations of MDA via autoxidation (0.001 nmol/ml). Fe^{3+} produced the highest concentration of MDA (0.044 nmol/ml). The Fenton Reaction treatment (10 µM Fe^{3+} + 20 mM H_2O_2) produced lower concentrations of MDA compared to Fe^{3+} treatment with only 0.019 nmol/ml. The Fenton Reaction treatment with 10 µM Fe^{3+} + 40 mM H_2O_2 and 10 µM Fe^{3+} + 80 mM H_2O_2 produced

0.0171 nmol/ml and 0.0170 nmol/ml of MDA, respectively. The Fenton Reaction treatment with 10 µM Fe^{3+} + 160 mM H_2O_2 produced 0.013nmol/ml. Finally, the 10 µM Fe^{3+} + 640 mM H_2O_2 produced the lowest concentration of MDA at 0.002 nmol/ml. Fe^{3+} elevated MDA production relative to the arachidonic acid only treatment as did the 10 µM Fe^{3+} + 20 mM H_2O_2 ($p<0.001$) MDA was not elevated relative to the arachidonic acid only treatment and the 10 µM Fe^{3+} + 20 mM H_2O_2 , 10 µM Fe^{3+} + 40 mM H_2O_2, 10 µM Fe^{3+} + 80 mM H_2O_2, or 10 µM Fe^{3+} + 160 mM H_2O_2 treatment ($p\geq0.289$). MDA production was higher however, between the 10 µM Fe^{3+} + 160mM H_2O_2 and 10 µM Fe^{3+} + 640 mM H_2O_2 treatment ($p<0.001$).

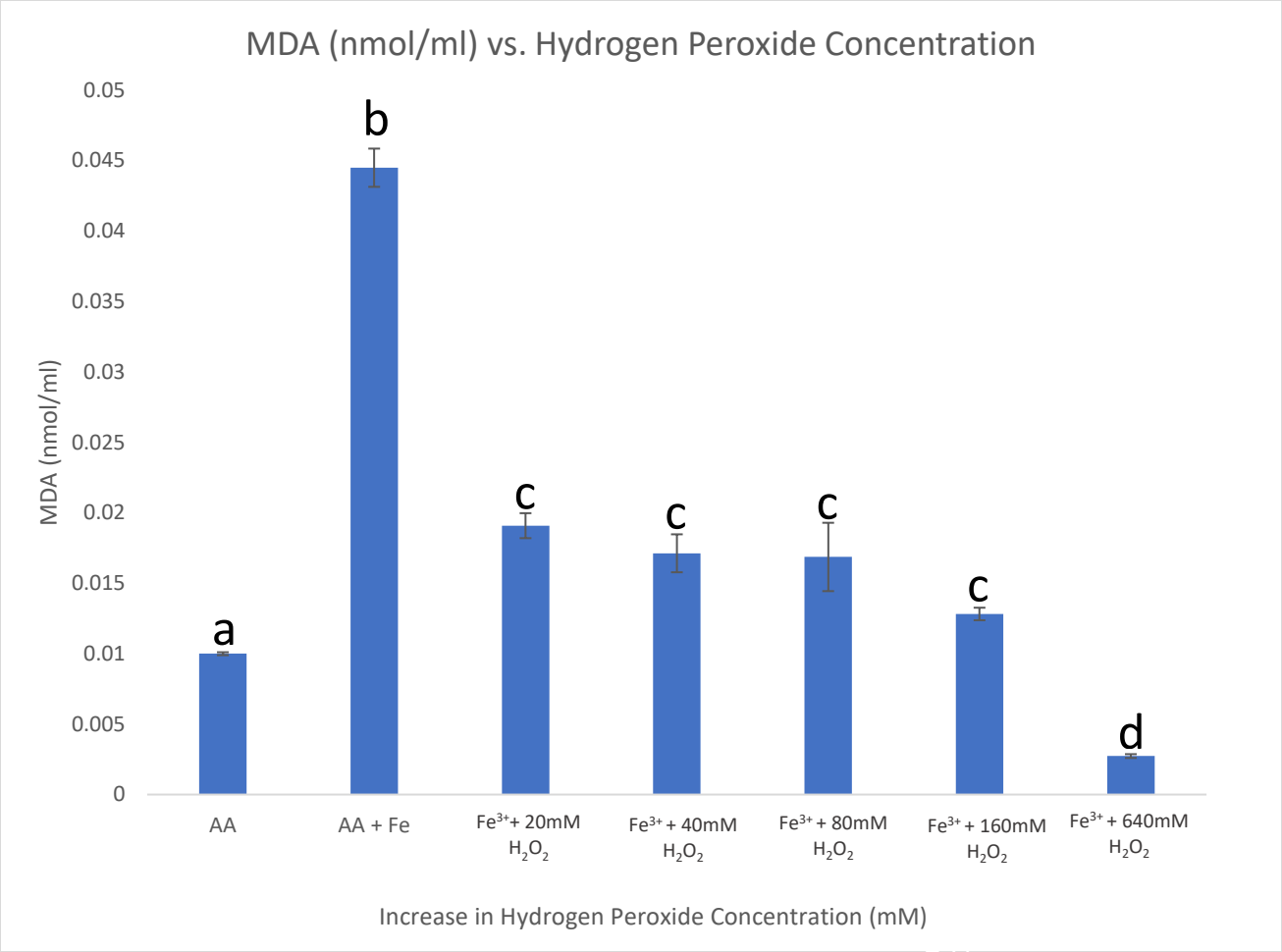


Figure 11. The arachidonic acid *in vitro* model was exposed to the Fenton Reaction (Fe^{3+} +H_2O_2) with increasing concentrations of hydrogen peroxide. The Fenton Reaction induced lipid peroxidation of arachidonic acid *in vitro* model and produced concentrations of malondialdehyde. Mann-Whitney statistical test was used, and lower-case letters indicate statistical significance between treatments at $\alpha<0.05$. Mean ± SEM and $n = 3$.

3.4 Fluorescence Intensity of TBA-MDA Chromogen

The fluorescence intensity of the TBA-MDA chromogen was monitored over 30 min duration while simultaneously being exposed to three treatments. 10 µM Fe^{3+}, Fenton Reaction

(10 µM Fe^{3+} + 20 mM H_2O_2), and 20 mM hydrogen peroxide only. During the 30 min, there was no change in the fluorescence intensity of TBA-MDA chromogen while being exposed to 10µM Fe^{3+}. However, during this time, there was a reduction (p<0.001) in the fluorescence intensity of the TBA-MDA chromogen exposed to 20 mM hydrogen peroxide treatment. Also, while the TBA-MDA chromogen was being exposed to the Fenton Reaction, there was a significant decrease in the fluorescence intensity over (p<0.001).

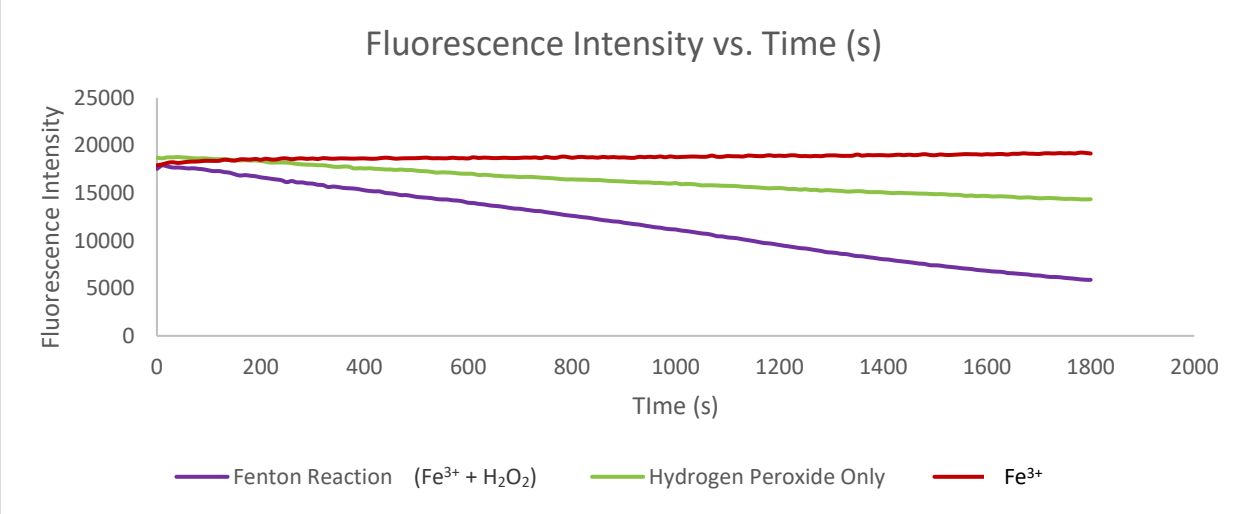


Figure 12. Time course of fluorescence intensity of TBA-MDA chromogen exposed to various treatments (hydrogen peroxide, Fe^{3+} and Fenton Reaction (Fe^{3+} + H_2O_2)), over a 30 min duration.

3.5 TBA-MDA Chromogen Exposed to Fenton Reaction with Decreasing Concentrations of Hydrogen Peroxide

The TBA-MDA chromogen was exposed to various treatments, with decreasing concentrations of hydrogen peroxide in the Fenton Reaction. Treatments included a hydrogen

peroxide treatment, an Fe^{3+} treatment, a 10 µM Fe^{3+} and 10 µM H_2O_2 treatment, a 10 µM Fe^{3+} and 100 µM H_2O_2 treatment, and a 10 µM Fe^{3+} and 1 mM H_2O_2 treatment, a 10 µM Fe^{3+} and 20 mM H_2O_2. There was no decrease (p=0.086) in the fluorescence intensity of chromogen while exposed to iron Fe^{3+} alone. There was a decrease in the fluorescence intensity of chromogen while exposed to hydrogen peroxide alone (p<0.001). There was no significant decrease in the fluorescence intensity of the 10 µM Fe^{3+} and 10 µM H_2O_2 and 10 µM Fe^{3+} and 100 µM H_2O_2 treatments compared to chromogen only control (p>0.079). Finally, there was a large decrease in the fluorescence intensity of the 10 µM Fe^{3+} and 1 mM H_2O_2 and 10 µM Fe^{3+} and 20 mM H_2O_2 treatments (p<0.001).

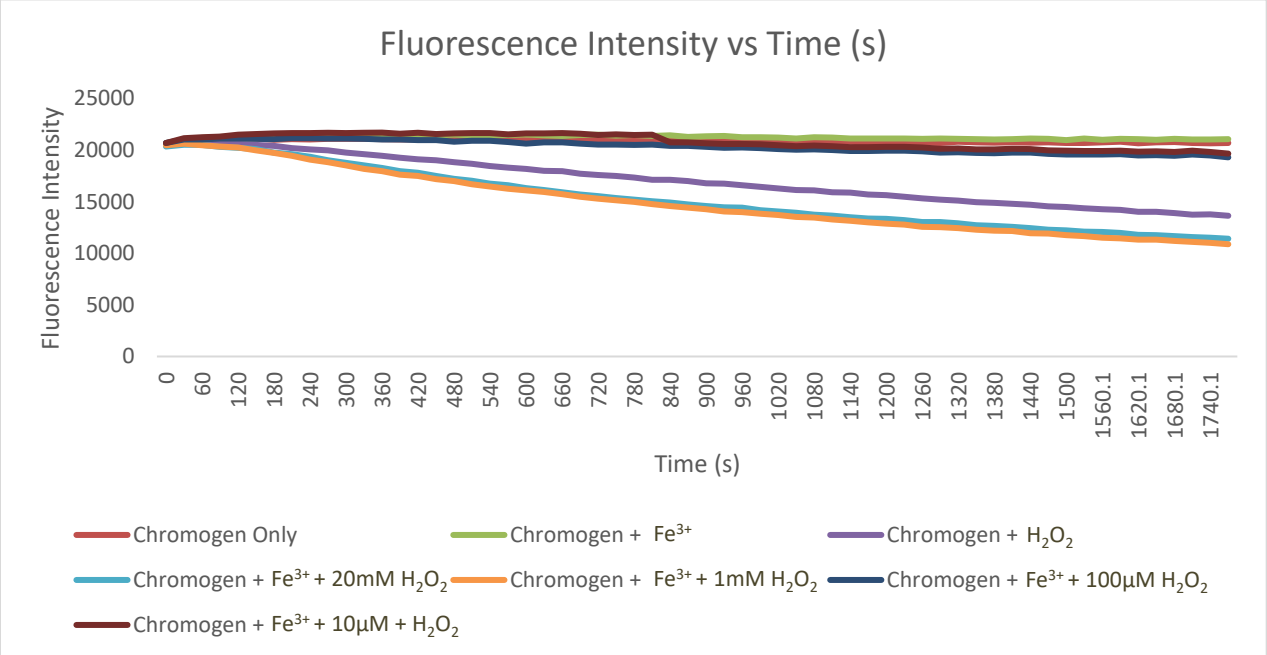


Figure 13. The fluorescence intensity of TBA-MDA chromogen was measured while being exposed to Fenton Reaction (Fe^{3+} + H_2O_2) with decreasing concentrations of hydrogen peroxide during a 30-min period.

3.6. Iron Chelators DIBI and DFP Reducing Lipid Peroxidation and MDA Concentrations

An arachidonic acid *in vitro* model was exposed to Fe^{3+} treatment to induce lipid peroxidation. Chelators were used to prevent the initiation of lipid peroxidation and consequently the production of MDA. The arachidonic acid alone produced 0.025 nmol/ml MDA by autoxidation while, the Fe^{3+} treatment produced the highest concentration of MDA, while the Fe^{3+} treatment (0.100 nmol/ml) elevated MDA production (p<0.001) relative to arachidonic acid alone. Both the DIBI (p<0.001) and DFP (p<0.001) iron chelators reduced lipid peroxidation relative to the Fe^{3+} only treatment, limiting MDA production to 0.023 nmol/ml and 0.042 nmol/ml of MDA. However, the DFP iron chelator produced more MDA than was produced in the presence of DIBI (p<0.001). MDA production in the presence of the DIBI chelator did not differ from the arachidonic acid only control (p=0.724). However, MDA concentrations were elevated relative to the arachidonic acid control despite the presence of DFP chelator (p=0.013).

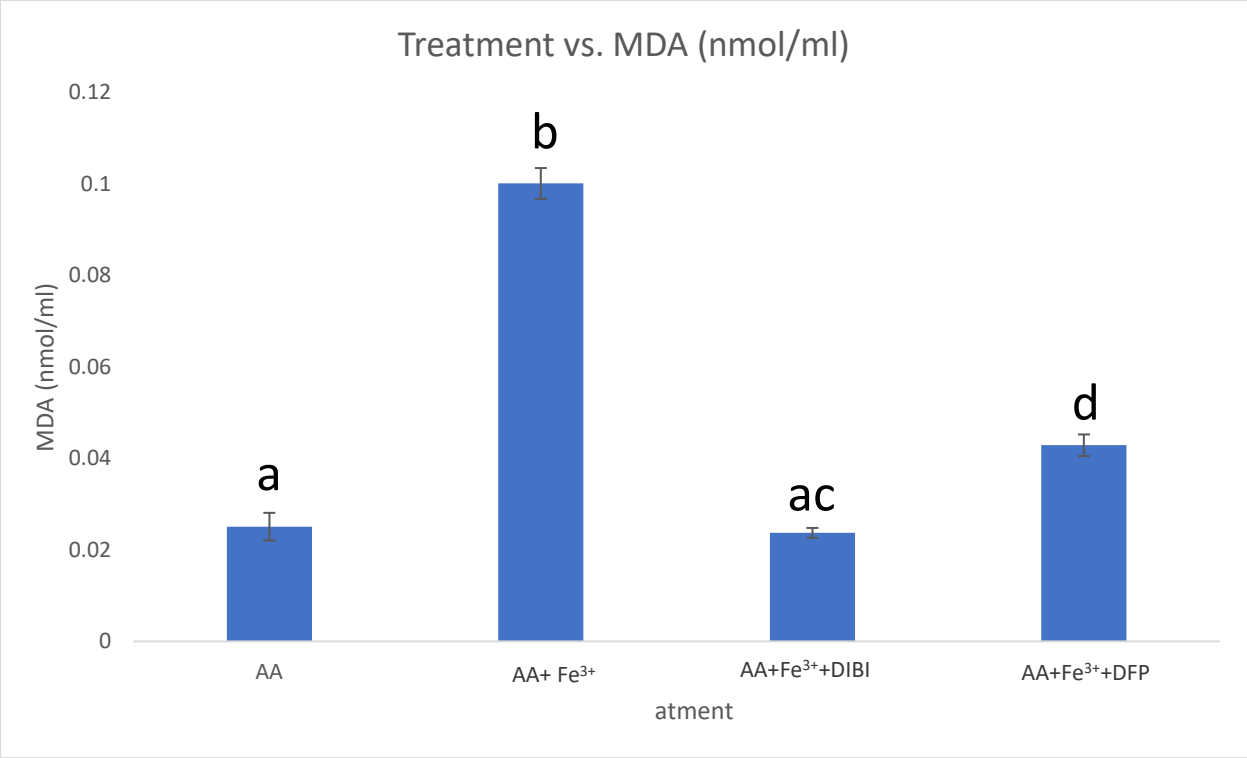


Figure 14. Iron chelators deferiprone (DFP) and DIBI were used to prevent lipid peroxidation of an arachidonic acid *in vitro* model. Fe^{3+} treatment was used to initiate lipid peroxidation and produced concentrations of malondialdehyde. Mann-Whitney statistical analysis test was used, and lower-case letters indicate statistical significance between treatments at $\alpha<0.05$. Mean ± SEM and $n = 3$.

4 Discussion

The main objective of this study was to compare the efficacy of DIBI and DFP chelators at reducing lipid peroxidation using a novel application of the TBARS assay employing an *in vitro* arachidonic acid model. Within this study, DIBI was more effective at reducing lipid peroxidation and MDA concentrations compared to DFP. Previous research suggested DIBI had a higher avidity for iron and that DIBI sequestered iron was less available for reactions compared

to DFP sequestered iron (del Carmen Parquet, 2018). DFP being a bidentate chelator, required three DFP chelators to bind one Fe^{3+} atom. Bidentate chelators were shown to not be as effective and the chelated iron continued to redox cycle and promote ROS generation (Hatcher *et al.,* 2009). DIBI is a multivalent chelator allowing it to bind iron more effectively than DFP which is a monovalent chelator (Chelation Partners, 2019). This could explain why there was still lipid peroxidation in the presence of the DFP chelator. DIBI chelator could not completely reduce MDA because there were pre-existing levels of MDA from PUFA autoxidation, the oxidation of a substance in the presence of oxygen (Smith, 1981). PUFAs, such as arachidonic acid can undergo autoxidation to produce low concentrations of lipid hydroperoxides (Porter, 1986). Lipid hydroperoxides then breakdown into MDA which can react with TBA in TBARS assay (Stanojević *et al.,* 2018). Regardless, DIBI was able to prevent new production of MDA in the *in vitro* arachidonic acid model.

Arachidonic acid was the *in vitro* model chosen to undergo lipid peroxidation. Typically, the more double bonds found within the PUFA, the higher production of lipid peroxidation, and consequently higher generation of MDA (Begin *et al*., 1988). PUFAs with more double bonds have more sites that can be oxidized, resulting in increased abstraction of hydrogen and lipid peroxidation initiation (Sun *et al.,* 2011). This was consistent with the concentrations of MDA produced when three different fatty acids were exposed to the Fenton Reaction (Figure 9). As predicted, arachidonic acid, with four double bonds, produced the highest concentrations of MDA. Linoleic acid with two double bonds, produced intermediate concentrations of MDA. Oleic acid with one double bond, produced the lowest concentration of MDA. Research by Yiin and Lin (1995) also found *in vitro* arachidonic acid produced significantly more MDA than *in*

vitro linoleic acid. Thus, arachidonic acid is an appropriate *in vitro* model to undergo lipid peroxidation.

Initially, the Fe^{3+} treatment induced higher concentrations of MDA compared to the Fenton Reaction treatment (Figure 10). With further research, it appeared that increasing the hydrogen peroxide concentration in the Fenton Reaction decreased the concentration of MDA produced. Similarly, Kornbrust and Mavis (1980) found that the Fenton Reaction ($Fe^{3+} + H_2O_2$) did not increase iron-induced liposomal peroxidation. In fact, at higher concentrations of hydrogen peroxide, lipid peroxidation was inhibited. This is consistent with the results found in this study (Figure 11). Although, liposomes were used in their study, their conformation includes lipids containing fatty acid components that were oxidized. Additional testing revealed that the TBA-MDA chromogen appeared to be degraded when exposed to the hydrogen peroxide and the Fenton Reaction treatments (Figure 12). This could explain why the Fenton Reaction treatment generated significantly less MDA than the Fe^{3+} only treatment (Figure 10). If the TBA-MDA chromogen is being degraded, the MDA concentration cannot be accurately quantified. More testing revealed that decreasing the concentration of hydrogen peroxide, decreased the degradation of the TBA-MDA chromogen So, increasing the hydrogen peroxide in the Fenton Reaction treatments increased the concentration of ROS formed, thus, it appears the TBA-MDA chromogen is degraded by ROS (Figure 13). Despite our findings, Repetto *et al.*, (2010), reported no problems using TBARS to quantify lipid peroxidation induced by the Fenton Reaction. Regardless, our data demonstrate the Fe^{3+} treatment alone can be used to generate MDA within the *in vitro* TBARS arachidonic acid model since experimentally it did not degrade the TBA-MDA chromogen. Further research is required to understand the conditions and

generated ROS which degrade the TBA-MDA chromogen within the arachidonic acid model system.

Within this study, Fe^{3+} was able to induce lipid peroxidation quantifiable by TBARS with stable chromogen production in an *in vitro* arachidonic acid model. Again, our experimental results contrast research conducted by Rao *et al*., (1978) who found that Fe^{3+} was unable to induce lipid peroxidation in arachidonic acid because there was no formation of TBA reactive compounds. Rather, Rao *et al*., (1978) reported only Fe^{2+} could generate TBA reactive compounds. Earlier research conducted by Wills (1965) revealed that several metals, including Fe^{3+} were able to oxidize unsaturated fatty acids. More recently, Yiin and Lin (1998) found that transition metals such as cadmium could significantly increase the production of MDA of an *in vitro* arachidonic acid model. In findings complementary to ours (Figure 14), the results of Yiin and Lin (1998) showed that arachidonic only produced low concentrations of MDA, whereas the arachidonic acid with the transition metal, cadmium, greatly increased the production of MDA. Ultimately, the greater preponderance of research is consistent with Fe^{3+} inducing lipid peroxidation in an *in vitro* arachidonic acid model, consistent with our work.

Many studies suggest lipid hydroperoxides are essential for Fe^{3+} to initiate lipid peroxidation (Gutteridge, 1984; Davies and Slater, 1987; Hatcher *et al*., 2009). However, based on the results of this study, I propose a novel mechanism to explain how Fe^{3+} can initiate lipid peroxidation. Fe^{3+}can abstract a hydrogen from the allylic bond found in a PUFA such as arachidonic acid and produce a lipid radical that becomes a lipid peroxy radical. The lipid peroxy radical continues to react with other PUFAs and the propagation of lipid peroxidation reaction continues (Figure 6). However, the peroxy radical is not potent enough to destroy the TBA-MDA chromogen, unlike the more reactive products (*i.e.* hydroxyl radical) of the Fenton Reaction.

Macfarlane (2001) also suggested a mechanism where iron abstracts a hydrogen from an unsaturated lipid, similar to Fe^{3+} abstracting a hydrogen from a PUFA. Davies and Slater (1987) found that only Fe^{2+} was able to oxidize lipid peroxides hydroperoxides, not Fe^{3+} and no peroxyl radical or lipid hydroperoxides were present. Although Davies and Slater (1987) found Fe^{3+} was not an effective oxidizer of lipid hydroperoxides, and thus would not have reacted with the pre-existing lipid hydroperoxides, Hatcher *et al*., (2009) more recently found Fe^{3+} could produce lipid radicals by reacting with lipid hydroperoxides. While lipid hydroperoxides, according to Hatcher *et al*., (2009) could react with Fe^{3+} to initiate lipid peroxidation, there are higher concentrations of PUFAs both within the *in vitro* arachidonic acid model, as well as with the cell membrane. Thus, Fe^{3+} may be more likely to interact with PUFAs before encountering lipid hydroperoxides. However, further research is needed to determine if lipid hydroperoxides are necessary for Fe^{3+} induced lipid peroxidation.

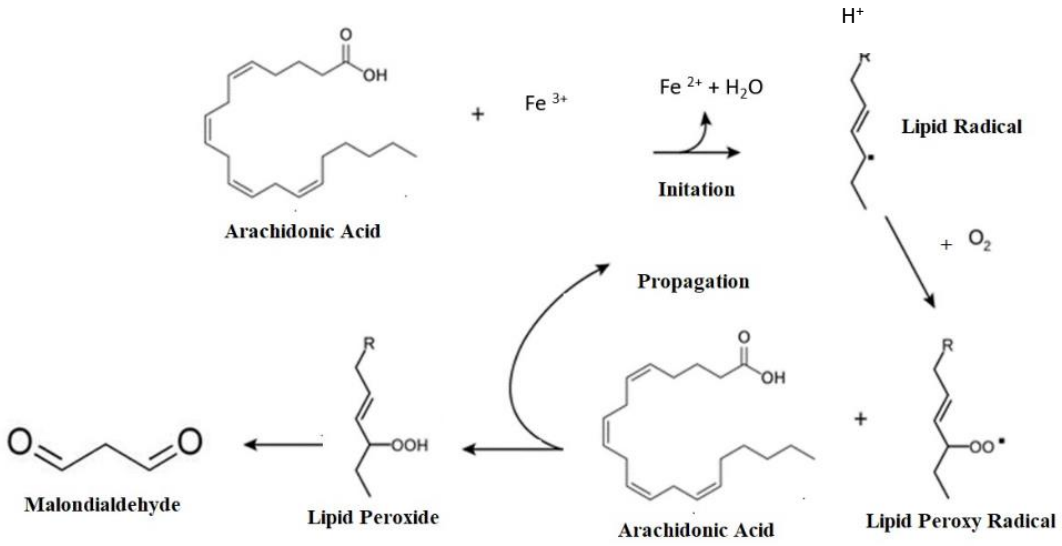


Figure 15. Arachidonic acid undergoing lipid peroxidation initiated by Fe^{3+}. Fe^{3+} initiates lipid peroxidation by abstracting a hydrogen from arachidonic acid. Fe^{3+} was reduced to Fe^{2+} and a lipid radical was formed. The lipid radical reacted with oxygen to form a lipid peroxy radical. The lipid peroxy radical reacts with other arachidonic acid molecules and propagates a chain reaction to produce more lipid peroxy radicals. Lipid peroxides can also be formed which then degrade to form malondialdehyde, a stable product of lipid peroxidation which can be quantified (Modified from Madhura, 2015).

5 Conclusion

In conclusion, this study compared the efficacy of Fe^{3+} chelators DIBI and DFP at reducing lipid peroxidation of an *in vitro* arachidonic acid model and investigated the novel application of the TBARS assay to quantify MDA. Lipid peroxidation was induced by the Fe^{3+} treatment and chelators were used to prevent the production of MDA. At equivalent binding capacities, DIBI was more effective at reducing MDA concentrations compared to DFP. Also, Fe^{3+} was sufficient to induce lipid peroxidation in the *in vitro* arachidonic acid model. Finally, a novel application of the TBARS was ineffective for quantifying lipid peroxidation induced by Fenton Reaction as the TBA-MDA chromogen was degraded during the TBARS assay. However, the TBARS assay could effectively quantify lipid peroxidation induced by Fe^{3+}, presumably as weaker ROS were generated than those generated by the Fenton Reaction. Further research is needed to understand the mechanism and species involved in TBA-MDA chromogen degradation, as well as those generated by the Fe^{3+} *in vitro* arachidonic model.

Iron chelators are an important tool for reducing oxidative stress associated with disease conditions (Arora *et al*., 2018). Promising research by Arora *et al* (2018) revealed the anti-inflammatory effects of DIBI chelator in non-infectious uveitis. Similarly, DIBI was effective at reducing inflammation caused by sepsis (Thorburn *et al*., 2017). Overall, DIBI can selectively bind iron, is non-toxic and biocompatible, which makes it a promising clinical candidate (Thorburn *et al*., 2017). However, significant research is required before DIBI can be used orally.

5.1 Future Considerations

The next step would be to research if iron chelators can prevent or reduce lipid peroxidation within a more complex model. Such models could include liposomes, human cells or a model organism such as zebrafish (*Danio rerio*). More complex models could change the efficacy of the iron chelators because of more interference other biomolecules (proteins, amino acids, lipids). As well, the Fe^{3+} and Fenton Reaction treatments might have different effects on complex models. The DIBI chelator could be further evaluated for its efficacy in inhibiting lipid peroxidation in liposomes and human cells. Also, zebrafish are an excellent model organism for studying human diseases due to their early development, transparency of embryos, high fecundity and short generation times (Dooley and Zon, 2000). Promising research by Nasrallah *et al.,* (2018) has shown that iron chelators could decrease iron within zebrafish larvae with no toxic effects. Also, research by Chen *et al.,* (2014) used iron chelators to prevent iron induced osteoporosis in zebrafish. So, zebrafish could be an excellent candidate for researching the efficacy of the DIBI chelator.

Further research is also required to prevent TBA-MDA chromogen degradation in TBARS assay. If MDA was isolated prior to the TBARS assay, the Fenton Reaction and hydrogen peroxide treatments could not degrade the TBA-MDA chromogen. Catalase, an enzyme which breaks down hydrogen peroxide, could be added to samples prior to TBARS assay. This could stop ROS production from the Fenton Reaction. Further research is also needed to determine if lipid hydroperoxides are necessary for Fe^{3+} induced lipid peroxidation. Sodium borohydride could be used to remove lipid hydroperoxides prior to exposure to the Fe^{3+} treatment (Kontush *et al*, 1996).

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