

The Effect of Deferoxamine and Ferrostatin-1 Treatments on Total ROS in HK-2 Cells

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The Effect of Deferoxamine and Ferrostatin-1 Treatments on Total ROS in HK-2 Cells

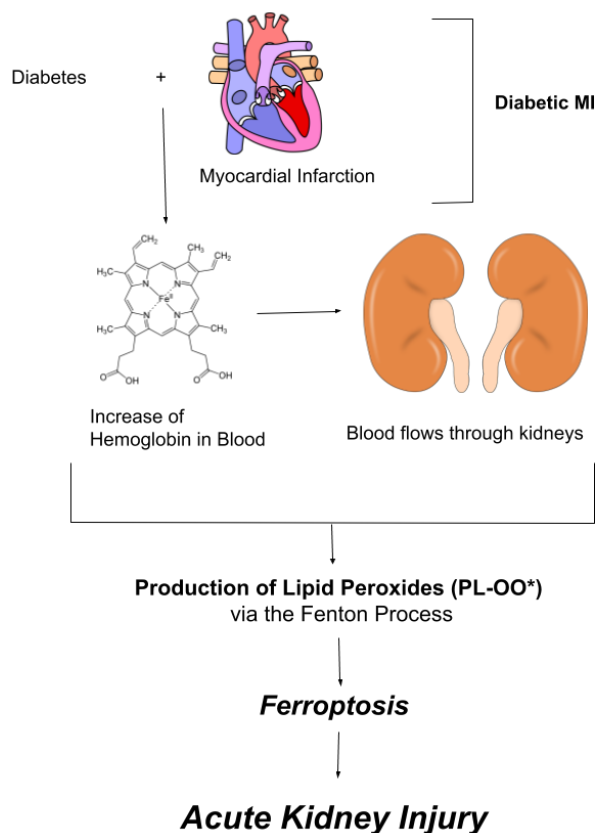
Abstract

Investigations indicate that diabetic patients with myocardial infarctions (MI) have an increased risk of acute kidney injury (AKI) post-MI. In diabetic MI, blood hemoglobin (Hb) rises, which induces the production of reactive oxygen species (ROS) that cause AKI through ferroptosis. Deferoxamine (DFO) and ferrostatin-1 (Fer-1) are treatments that reduce ROS through iron-chelation or the catalysis of radical reduction, respectively. This study investigates how ROS levels in Proximal Tubular Cells (HK-2) change with concentration adjustments of Fer-1 and DFO, as well as whether a mixture of DFO and Fer-1 at 1 μ M causes a synergic reduction of ROS. The results indicate that DFO, treated at 1 μ M, 10 μ M, and 100 μ M, had a dose-dependent reduction of ROS but did not achieve a significant reduction of ROS ($p > 0.05$) at 1 μ M. Conversely, DFO 1 μ M significantly increased ROS in the second experiment, indicating possible prooxidant interactions of DFO at low concentrations. Fer-1, treated at 1 μ M, 5 μ M, and 10 μ M, indicated a dose-dependent response until 5 μ M. However, there was no significant reduction in ROS between 5 μ M and 10 μ M due to the unsaturation of Fer-1 past 5 μ M. When Fer-1 and DFO were mixed at 1 μ M, the ROS was not significantly different from Fer-1 1 μ M, so there was no synergic effect ($p > 0.05$). Hence, future treatments of Fer-1 and DFO should be done independently, DFO should be treated at concentrations of at least 10 μ M, where there is a significant reductive effect, and Fer-1 should be treated up to 5 μ M, where there is maximal ROS reduction.

Introduction

Coronary heart disease is the leading cause of death in America and heart disease found in 48% of the US population and myocardial infarctions (MI) have become extremely common (Roger, 2007; American Heart Association, 2019). Besides heart injury, MI has additional implications for diabetic patients. There is evidence that Diabetic MI causes myocardial hemorrhage, which leads to increased heme, or iron-binding proteins like Hemoglobin (Hb), in the bloodstream (Gupta, 2020). Increased heme from diabetic MI induces reactive oxygen species (ROS) production in the kidneys, causing ferroptosis or iron-induced cell death (Gupta, 2020) (see Figure 1). The process of ferroptosis in the kidneys is found to be a major driver of Acute Kidney Injury (AKI), explaining why diabetes is a key predictor for AKI post-MI, according to the Journal of the American Heart Association (Dixon, 2012; Hu, 2019; Tonnus, 2017; Marenzi, 2018).

Figure 1.
Mechanism of AKI Incidence Post-Diabetic MI



In ferroptosis, iron (Fe) from hemoglobin (Hb) induces ROS production through the fenton process (see Figure 1). Hb's Fe^{+2} catalyzes the production of lipid alkoxyl radicals (PL-O^*) from lipid hydroxides (PL-OH). PL-O^* molecules spontaneously react and turn into lipid peroxides (PL-OO^*), which lead to ferroptosis and AKI (Miotto, 2020; Dixon, 2012). A treatment that inhibits any of these steps could effectively reduce ferroptosis and AKI.

Since AKI has a 40-50% mortality rate, investigating the effects of deferoxamine (DFO) and ferrostatin-1 (Fer-1) treatments on ROS reduction could save many lives (Workeneh, 2020). Deferoxamine is an iron chelator which reduces ROS by scavenging the iron in the cell (Deferoxamine, 2020). By scavenging the iron, the production of PL-O^* , the initiation step, will decrease and prevent the production of lipid peroxides (PL-OO^*), decreasing ROS and ferroptosis (Zille, 2008). Ferrostatin-1 functions as a catalyst radical that turns a PL-O^* into PL-OH by converting the oxygen radical group into a hydroxyl group (see Figure 9) (Miotto, 2020). There has been evidence for the effectiveness of Fer-1, for Folic acid-induced AKI that was pretreated with ferrostatin-1 presented with significantly less tissue damage and better renal function (Martin-Sanchez, 2017). Similarly, it has also been found that cell viability increases significantly with treatments of DFO and Fer-1 to heme-treated cells (Zille, 2017).

Although cell viability and histological studies have been done, this study investigates the specific response of ROS in HK-2 cells, or proximal tubular kidney cells, as concentrations of Fer-1 and DFO change, deepening the understanding of how different treatments of DFO and Fer-1 interact with Hb and Hb-induced ROS. HK-2 cells, or proximal tubular kidney cells, were used and treated in this experiment to ensure the significance of the findings in the larger premise of Diabetic MI induced AKI research since damage to HK-2 cells causes AKI.

Hypothesis

When deferoxamine and ferrostatin-1 are treated at increasing concentrations, there will be a dose-dependent reduction of ROS between all three groups. Furthermore, when both reagents are mixed at $1\mu\text{M}$, a synergic reduction in ROS will be observed, where the combined solution of Fer-1 and DFO will produce a larger reduction in ROS than the individual Fer-1 and DFO solutions alone.

Methods

The method to group HK-2 cells, treat HK-2 cells, and measure the total ROS in the HK-2 cells involved four steps: thawing cells, seeding cells, treating cells, and measuring results. Thawing the cells is when the cells are melted from ice and plated for the first time (for the first cell passage). Seeding cells involve a fully confluent plate (100% filled with cells) split to add cells to a 96-well plate for treatment and to another plate for future use. Treating cells is the process of preparing and applying the experimental reagents for the cells. Measuring the results involves setting up the fluorescence machine to detect the total ROS.

Thawing Cells

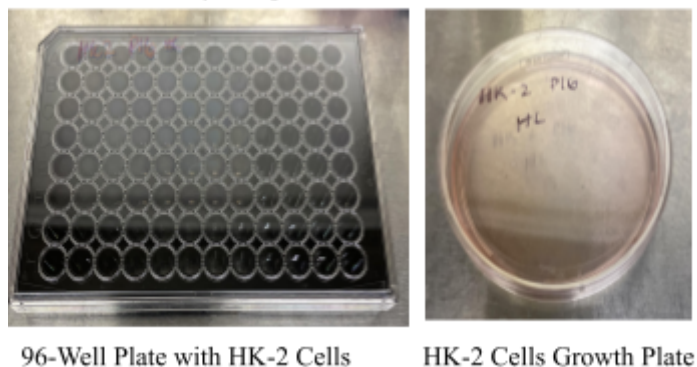
Thawing cells is a simple process where an aliquoted, frozen sample of the HK-2 cell line is melted and plated for further growth and use. In order to rapidly neutralize the melted freezing medium, the hood was prepared with Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and a cell plate. Next, the frozen aliquoted sample was removed and put into a 37°C water bath to be melted. When a sliver of ice remained, the sample was taken to the hood. After the ice melted, the sample was added to the plate with DMEM media and placed

in the incubator. The following day, a media change was done to remove any of the DMSO, a toxic cryoprotectant from the frozen cells, from the medium. Thawing cells is only required for the first plating of cells. For the rest of the experiments, the cells will be reseeded into new plates.

Seeding Cells

First, the full confluency of the cells was verified using a light microscope. If the cells were fully grown, then I proceeded to split the cells. The cells were first washed with PBS solution to remove debris. After removing the PBS, 1 mL of trypsin was added and the cells were incubated for 3 minutes to suspend the cells. After 3 minutes, the plate was tapped against the glass of the hood to lift remaining cells and then 4 mL DMEM with 10% FBS was added to neutralize the solution. A 10mL pipette was used to spread the media to suspend and collect remaining cells.

Figure 2.
Cell Plates Used for Experimentation



96-Well Plate with HK-2 Cells

HK-2 Cells Growth Plate

Cell Plate for Future Use

1 mL of the 5mL of cells was transferred to another cell culture dish and 9mL of DMEM with 10% FBS was added. The plate was tilted to spread the cells and was incubated for 5-7 days before it reached full confluence for reseeding.

96-Well Plate Preparation

In order to prepare the 96-well plate, a 25mL plastic tray is used to prepare the cells. 4 mL of the remaining cells were mixed thoroughly with 6 mL DMEM with 10% FBS in the tray. Then, a clear-bottomed 96-well plate was brought into the hood and cells were added to the wells using a multi-headed pipette (see Figure 3). The cells were then tilted at least 20 times on both the horizontal and vertical axis to spread the cells evenly. The cells were then placed in the incubator for use the next day.

Treating Cells

If the cells were spread evenly in each well with similar cell quantity for each well, then we continued with experimentation. Initially, media was removed from cell wells, PBS was added into a 25mL tray, and 100uL of PBS was added to experimental wells using a multi-headed pipette. Next, PBS was removed from wells and from the 25mL tray with a vacuum. 3 mL of ROS Buffer and 2.5uL of the 1000x ROS labeling solution were mixed thoroughly in the empty 25mL tray. 100uL of the solution was added to the experimental wells of the 96-well plate. The plate was placed into the incubator for 45 minutes. During the incubation time, the treatment samples were prepared (Reactive Oxygen Species (ROS) Detection Assay Kit, 2019).

Preparing Treatment Solutions

Based on previous calculations, the treatment concentrations of the ferrostatin-1 (Fer-1) and deferoxamine (DFO) reagents were prepared. Sequential dilution was used to prepare very low concentrations. After preparing the diluted solutions of Fer-1 and DFO, Hb was added to the solutions except for the control and the Hb 5 μ M solution for the Hb column was prepared. After the 45 minutes were over, the labeling solution was removed from the plates and 100 μ L of the treatment solutions were added to their respective wells. The plate was placed back into the incubator and was measured at 1 hour and 24 hours after treatment.

Measurement

The treated plate was taken to a machine that can fluorescently detect 96-well plates. The machine was set to a clear-bottom setting and the wavelength of detection was set to 495nm/529nm for Ex/Em. This was the wavelength that corresponded with the ROS label in the total ROS kit used. The relative fluorescence from each well quantifies the relative concentrations of ROS for each well and is used to compare the amount of ROS between groups.

Methodology

The use of the Total ROS measuring kit is a valid method for the measurement of ROS because the fluorescence measured directly targets that of the label bound to ROS. In order to verify their product, a study was done with an experimental control, which induces ROS, H₂O₂, which also induces ROS, a positive control, a negative control, and an assay buffer. The results were reliable and consistent with previous scientific research (Reactive Oxygen Species (ROS) Detection Assay Kit, 2019).

Statistical Analysis

The Analysis of Variance (ANOVA) test and the Turkey Cramer test were used to determine whether significance was achieved between groups. A p-value of less than 0.05 was deemed significant for the purposes of the results. Exact p-values can be found in the appendices.

Materials

The materials used in this experiment include ROS label and ROS buffer from a Total ROS kit, a fluorescence detector that is suited for measuring 96-well plates, a cell growth dish, clear-bottom 96-well plates, electronic pipettes with 10mL glass tips, micropipettes (100 μ L, 1000 μ L, 10 μ L) with respective micropipette tips, a cell incubator, a microscope to check the cells, and a water bath.

Figure 3.
Pipettes Used in Experimentation



Multi-headed Pipette

Pipette for larger volumes

Results

Experiment 1: Testing Dose-Dependent Response

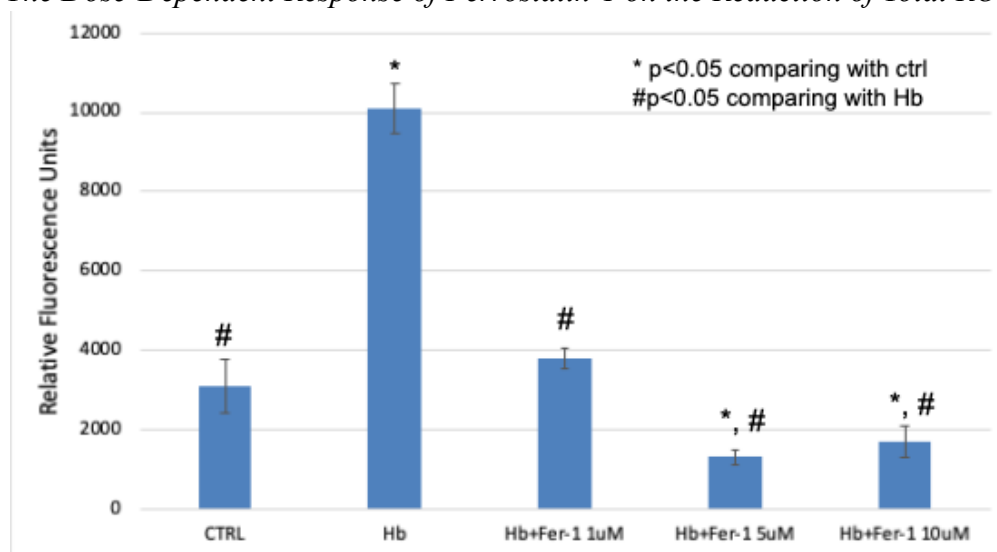
Ferrostatin-1 Treatment

Ferrostatin-1 (Fer-1) is confirmed to be an effective reducer of ROS because at concentrations of 1 μ M, 5 μ M, and 10 μ M, the reagent created a statistically significant reduction in ROS at both 1 hour and 24 hours (see figures 4 and 5). There was a dose-dependent response when the concentration of Fer-1 increased from 1 μ M to 5 μ M at 1 hour and 24 hours ($p < 0.05$). However, there was no significant ROS-reducing effect found by increasing the concentration of Fer-1 from 5 μ M to 10 μ M at 1 hour and 24 hours ($p > 0.05$). On the other hand, the average ROS slightly increased with an increased fluorescence of 364 RFUs at 1 hour and 3407 RFUs at 24 hours between Fer-1 5 μ M and Fer-1 10 μ M. The insignificance between the groups may be a result of the unsaturation of the catalyst Fer-1 after 5 μ M, so increasing the amount of Fer-1 after 5 μ M will cause an insignificant change in the reaction rate, meaning an insignificant change in ROS. Hence, the hypothesis that there will be a dose-dependent response at all 3 concentrations was not supported since there was no reduction in ROS between 5 and 10 μ M Fer-1.

Although there was a reduction effect for Fer-1 at both 1 hour and 24 hours, the effect diminishes at 24 hours. This is shown by how the percent of Hb's ROS increased dramatically between 1 hour and 24 hours, illustrating how the difference between the positive control and the treatments shrunk. For Fer-1 1 μ M, at 1 hour, the percent of the Hb's ROS was 37.39% while at 24 hours, it was 84.30%. Similarly, for Fer-1 5 μ M, at 1 hour, the percent of the Hb's ROS was 12.96% while at 24 hours, it was 50.99%. Lastly, for Fer-1 10 μ M, at 1 hour, the percent of the Hb's ROS was 16.56% compared to 60.43% at 24 hours. Since Fer-1 can not completely scavenge all radicals and Fer-1 removes radicals at a constant rate, as time progresses, the unaccounted radicals will accumulate and make the ROS removed from Fer-1 less significant with an increasing Total ROS (See Figure 11 and discussion).

Figure 4.

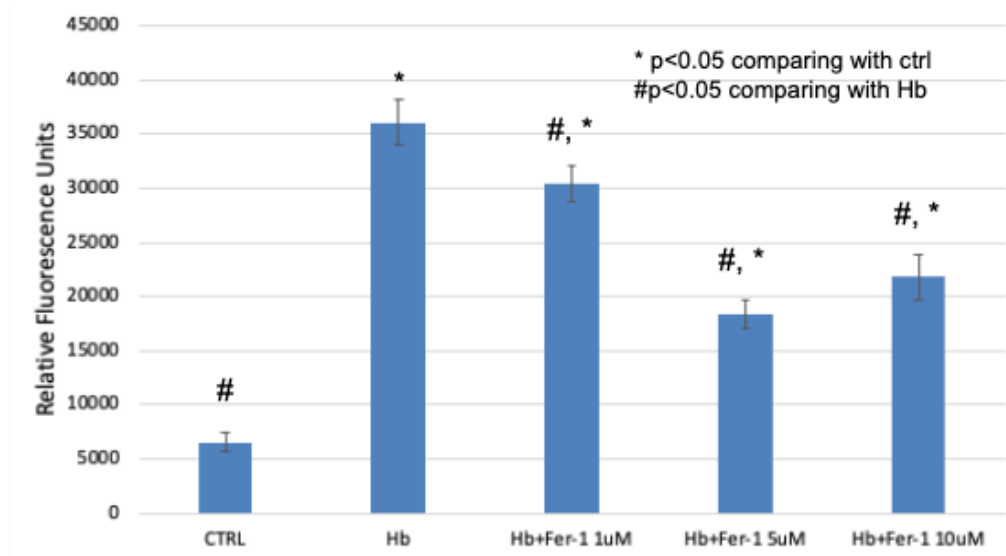
The Dose-Dependent Response of Ferrostatin-1 on the Reduction of Total ROS at 1 hour



Hb at 5 μ M, Fer-1=Ferrostatin-1. Averages for each group are shown. The error bars represent the standard deviation for each group.

Figure 5.

The Dose-Dependent Response of Ferrostatin-1 on the Reduction of Total ROS at 24 hours



Hb at 5 μ M, Fer-1=Ferrostatin-1. Averages for each group are shown. The error bars represent the standard deviation for each group.

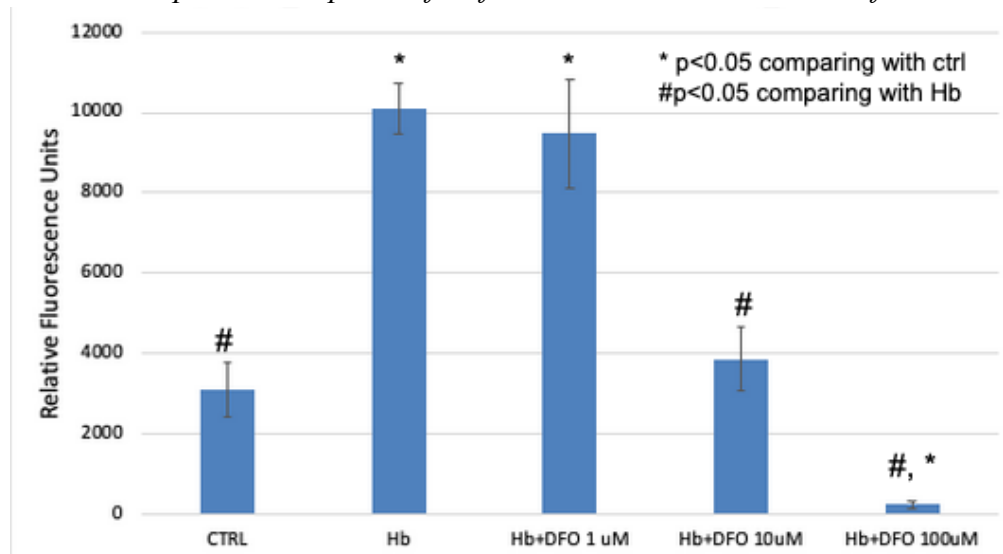
Deferoxamine Treatment

Deferoxamine (DFO) exhibits a dose-dependent response between the three concentrations in the reduction of ROS at both 1 hour and 24 hours of incubation although there is a stronger response at 1 hour (see figures 6 and 7). According to an Analysis of Variance (ANOVA) between groups, the p value is less than 0.05 between all three doses of DFO for both 1 hour and 24 hours of treatment but there is no exhibition of a significant reduction in ROS for DFO 1 μ M ($p>0.05$) for 1 and 24 hours. For the 24 hours testing of DFO 1 μ M, although the difference is insignificant ($p>0.05$), it is counterintuitive that DFO can cause an increase in ROS as compared to Hb, the positive control. However, research has found some scenarios in which DFO may cause an increase in ROS, such as DFO's interaction with nitrates and nitrites (refer to the discussion section). In addition, this same finding, but with significance, was found in the next experiment as well, indicating that DFO may have a significantly negative impact on ROS at 1 μ M after 24 hours. Because of the absence of a significant reductive effect of the 1 μ M concentration of DFO, the hypothesis was not supported because although there was a dose-dependent response between concentrations, significance with Hb was not achieved for DFO 1 μ M.

Similar to Fer-1, the strength of the dose-dependent response greatly decreased between 1 hour and 24 hours. In the experiment, DFO 1 μ M went from having 93.76% of the ROS as compared to Hb to having 9.12% more ROS than Hb. Similarly, DFO 10 μ M had 38.14% of Hb's ROS at 1 hour while at 24 hours, DFO 10 μ M had 78.55% of Hb's ROS. Lastly, for DFO 100 μ M, its ROS was 2.18% of Hb's ROS at 1 hour while at 24 hours, its ROS was 24.23% of Hb's ROS. The drastic increase in percent ROS as compared to Hb for the 3 experimental groups indicates that the efficacy of the drugs decreases substantially between 1 and 24 hours due to the saturation of iron in the DFO molecules (see discussion).

Figure 6.

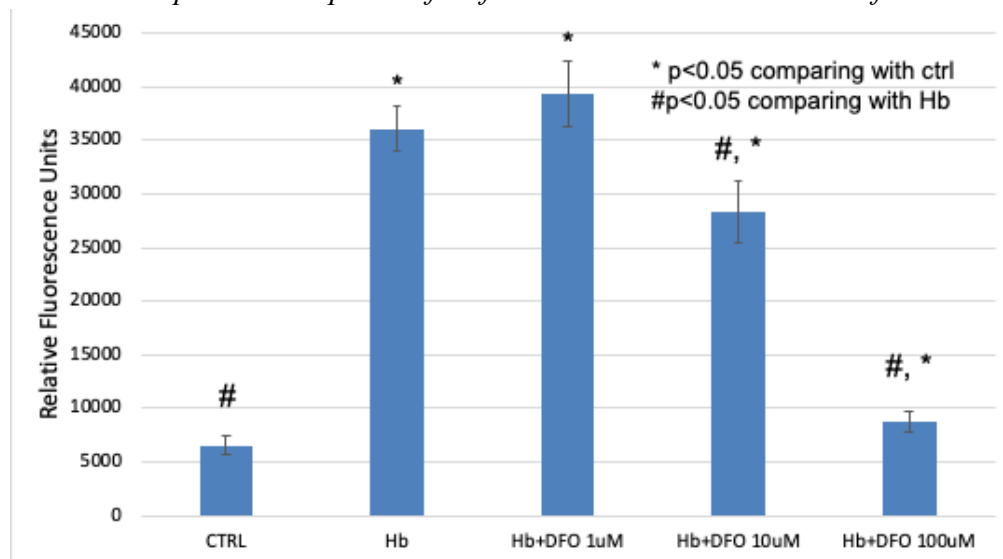
The Dose-Dependent Response of Deferoxamine on the Reduction of Total ROS at 1 hour



Hb is at 5 μ M, Deferoxamine=DFO. Averages for each group are shown. The error bars represent the standard deviation for each group.

Figure 7.

The Dose-Dependent Response of Deferoxamine on the Reduction of Total ROS at 24 hours



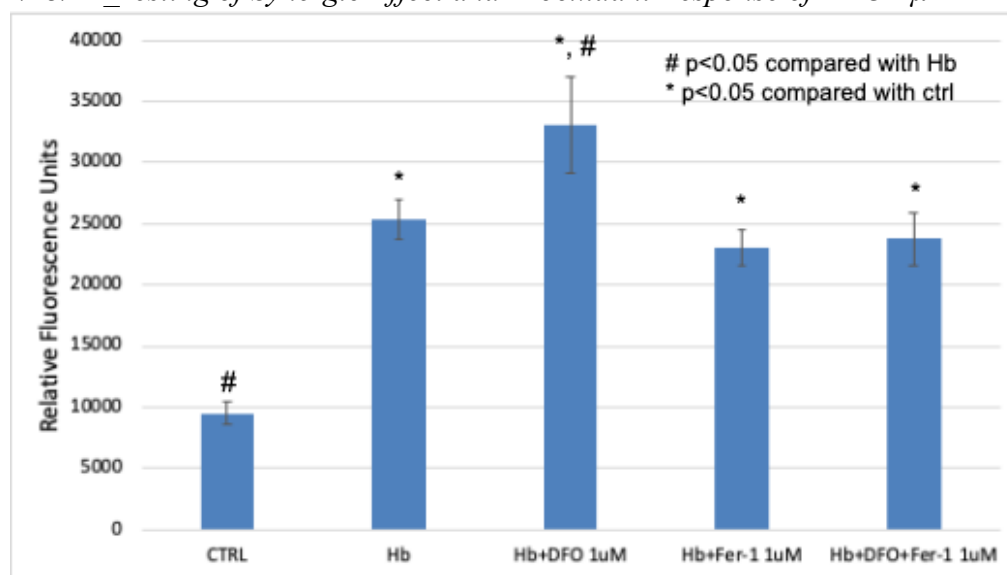
Hb is at 5 μ M, Deferoxamine=DFO. Averages for each group are shown. The error bars represent the standard deviation for each group.

Experiment 2: Testing of Synergic Effect and Prooxidant Response of DFO 1 μ M

This experiment was designed to discover whether a combination of DFO 1 μ M and Fer-1 1 μ M will result in a greater response than DFO or Fer-1 individually, or in other words, whether there would be a synergic effect. From the data presented, the hypothesis can be refuted, for it is manifest that this combination had no synergic effect on the reduction of ROS since the combined solution of DFO 1 μ M, Fer-1 1 μ M, and Hb had a p-value of 0.998 as compared to the Hb+Fer-1 group. Hence, there is an extremely negligible change of any synergic effect. Although DFO mainly has an antioxidant effect by scavenging iron, it is now being discovered that DFO can have prooxidant properties when it interacts with certain molecular species, such as nitrates and nitrites. At 1 μ M after 24 hours, it seems like DFO's prooxidant interactions overshadow DFO's antioxidant iron chelating effect, causing a significant raise in ROS with a p-value of 0.01, meaning that there is a 99% chance that the increase in ROS was not due to experimental variation.

Figure 8.

1/13/21_Testing of Synergic Effect and Prooxidant Response of DFO 1 μ M



Hb is at 5 μ M, Deferoxamine=DFO, Ferrostatin-1=Fer-1. Averages for each group are shown. The error bars represent the standard deviation for each group.

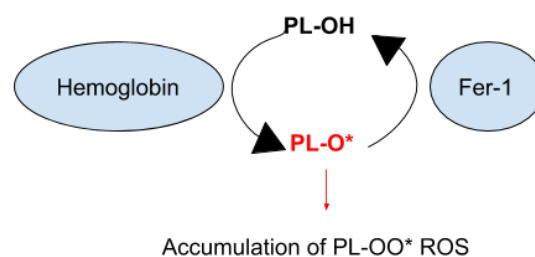
Discussion

Experiment 1: Testing Dose-Dependent Response

Ferrostatin-1 Treatment

Ferrostatin-1 (Fer-1) exhibited a dose-dependent response to the reduction of ROS between concentrations of 1 and 5 μ M ($p < 0.05$) while it did not see any response from a change from 5 μ M to 10 μ M ($p > 0.05$) for both 1 and 24 hours. This

Figure 9.
ROS-Reducing Mechanism of Fer-1



Fer-1 scavenges radicals and reduces ROS accumulation

dose-dependent reduction in ROS between 1 and 5 μM is a result of Fer-1's PL-O* radical scavenging ability (see Figure 9) and the saturation of the catalyst at 1 and 5 μM . A possible explanation for the lack of a reductive effect with the increase of concentration from 5 to 10 μM is that the catalyst is not saturated past 5 μM and additional Fer-1 catalyst can not increase the reaction rate, leading to no significant decrease in ROS compared to 5 μM . Because there was no reduction in ROS between 5 and 10 μM , the hypothesis is refuted, for a dose-dependent response was not observed for all 3 concentrations.

Deferoxamine Treatment

Deferoxamine has exhibited a dose-dependent response between its groups due to its property as an iron-chelator that binds to iron at a 1:1 molar ratio (Lu, 2008). As the concentration increased from 1 to 10 μM and from 10 to 100 μM , there was a significant decrease in ROS ($p < 0.05$). This is because as the concentration of deferoxamine increases, the more iron from Hb can be bound to the deferoxamine compound, causing a reduction in ROS since there is less Hb to initiate alkyl oxide radical formation (PL-O*) from lipid hydroxides (PL-OH) (Miotto, 2020).

However, there was no significant difference ($p > 0.05$) between DFO 1 μM and Hb at 1 hour and 24 hours. Interestingly, the ROS increased slightly at 24 hours. This finding at 24 hours indicates the presence of other competing prooxidant interactions that was not considered prior to experimentation (more details in "Deferoxamine Elevation of ROS" below).

Changes in Reducing Effect of Fer-1 and DFO between 1 and 24 hours

Between 1 hour and 24 hours, there was a diminished effect of DFO and Fer-1 on decreasing ROS. This was seen by very large rises in the percent of Hb's ROS values for all concentrations (see tables 5 and 6). For Fer-1, this increase is because at every concentration, more ROS is produced than scavenged. Since Hb's Fe^{+2} is always producing ROS, a certain percentage will not be accounted for. Over time, those unaccounted radicals will accumulate and make Fer-1's constant reductive effect less significant as Total

Figure 10.
DFO's Mechanism of ROS Reduction

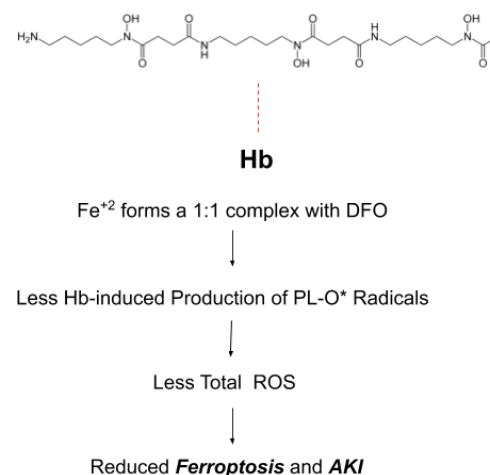
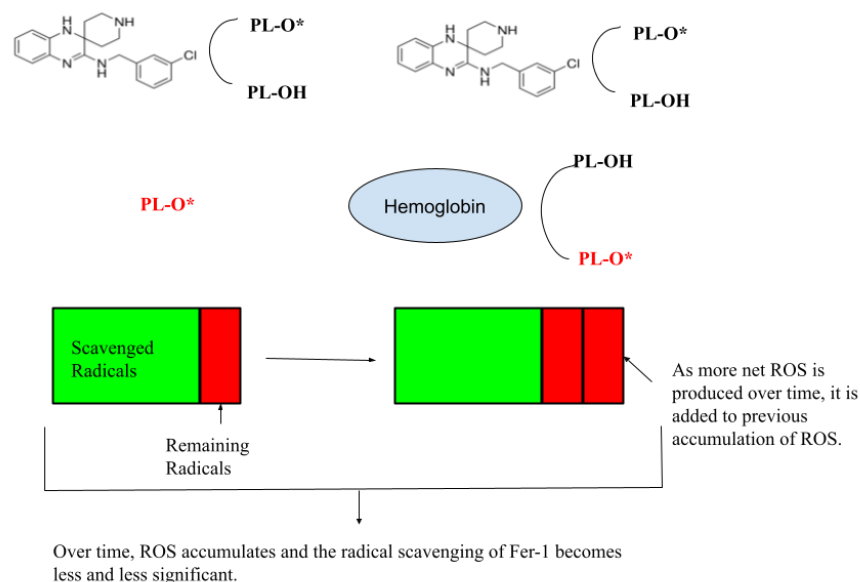


Figure 11.
Diminishing Effect of Fer-1 on Reducing ROS



ROS is elevated and the rate of ROS production from Hb is the same (see diagram on right).

With regards to DFO's diminished effect between 1 and 24 hours, because DFO can not bind to multiple Fe^{+2} ions from Hb, there is a limit of the ROS-reducing power that DFO has at any concentration. When all the DFO molecules are saturated with Hb, the remaining unbound Hb continues to initiate the production of alkyl oxide radicals (PL-O^*) and the gap between Hb and the Hb+DFO treatment decreases.

Experiment 2: Testing of Synergic Effect and Prooxidant Response of DFO 1 μM

Deferoxamine Elevation of ROS

Although deferoxamine (DFO) mainly serves a role in reducing ROS by chelating Fe^{+2} from Hemoglobin (Hb), DFO can also have prooxidant effects as well, as manifested by the significant rise in ROS for DFO 1 μM as compared to Hb ($p=0.01$).

Even though it's rare, this phenomenon has been found by other researchers as well (Francisco, 2010; Klebanoff, 1989; Lu, 2008). For example, Francisco, a researcher in the Federal University of Ouro Puerto in Brazil states that when administering DFO to 24 mice, the DFO was found to increase oxidative stress on proteins and cause the formation of NOS, or Nitrogen Oxidative Species like NO_2^* , which also has the ability to indirectly induce ROS, according to Zhao at Baylor University (Francisco, 2010; Zhao, 2007). This finding corroborates with Lu at

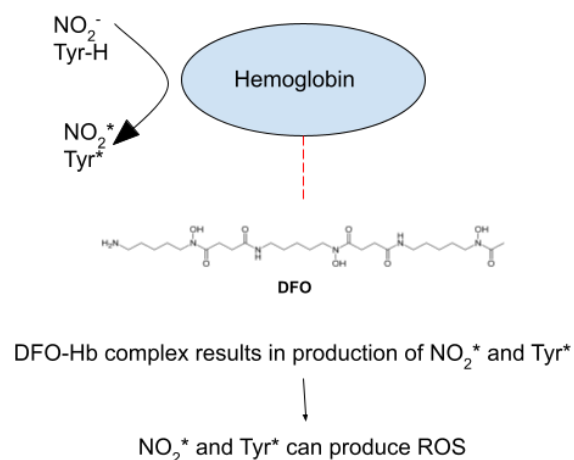
Huazhong University, who states that heme forms a novel intermediate with DFO, evidenced by changes in absorbance, and that this intermediate catalyzes the formation of NOS from nitrates/nitrites and caused increased oxidative damage to cellular proteins. As previously stated, according to Zhao, production of NOS may secondarily produce ROS as well, which might have accounted for the increase in ROS for DFO 1 μM found at 24 hours for both experiment 1 and 2 (Zhao, 2007).

With regards to the direct production of ROS, Lu states that the production of ROS from DFO has been found in interactions with paraquat, ferrous iron, and ascorbate (Lu, 2008). It might seem counterintuitive that DFO can both have an antioxidant and prooxidant effect for ferrous iron, but when interacting with Fe^{2+} , DFO can oxidize Fe^{2+} to Fe^{+3} , as seen in Klebanoff's study, and induce the production of ROS (Klebanoff, 1989).

At higher concentrations, prooxidant effects can be counteracted strongly by storing enough iron to reduce the Fenton process and production of radicals more than DFO's side reactions produce. However, at low concentrations of DFO, the indirect production of ROS through DFO's NOS production and Fe^{+2} oxidation might outpace its antioxidant properties since lower concentrations scavenge less iron. Although this explanation is plausible, this explanation requires further investigation of whether NOS production increases from DFO treatment in HK-2 cells and whether Fe^{+3} concentration rises in HK-2 cells.

Figure 12.

ROS-Increasing Mechanism of DFO



Testing of Synergic Effect

When DFO and Fer-1, at 1 μ M each, were combined, there was a p-value of 0.998 between the mixture and Fer-1, indicating that there was minimal change and there is a 0.002% chance of the difference carrying significance. This finding indicates that DFO had a negligible effect on ROS in the mixture and was overshadowed by the more significant decrease of ROS by Fer-1. From these results, it can be concluded that the antioxidant with a faster reaction rate determines the reduction effect with no presence of synergy.

Conclusion

The data collected in experimentation still suggests a general dose-dependent model of ROS-reduction from DFO and Fer-1; however, the model has limitations at low concentrations of DFO and concentrations past 5 μ M for Fer-1.

Fer-1 exhibited a dose-dependent ROS-reduction model for concentrations between 0 to 5 μ M ($p < 0.05$) with no dose-dependency between 5 and 10 μ M ($p > 0.05$). A possible explanation for this is that after 5 μ M Fer-1, the Fer-1 becomes unsaturated, so increasing the concentration of the catalyst will result in no change in reaction rate. Hence, with the same reaction rate, the amount of ROS reduction will be the same.

Between 1 and 10 μ M of DFO and 10 and 100 μ M DFO, there is a dose-dependent reduction of ROS ($p < 0.05$), indicating how the dose-dependency of DFO at the high end of concentrations holds well. After 1 hour of 100 μ M DFO treatment, the ROS was reduced significantly below the control, which had no treatment of Hb, indicating that the dose-dependent response model at high concentrations will continue to hold even as the ROS approaches the control ROS. However, at low concentrations, DFO does not follow a dose-dependent reduction of ROS. Instead, ROS was raised after 24 hours of DFO 1 μ M treatment with significance in Experiment 2 ($p = 0.01$). This finding alludes to the presence of DFO's prooxidant interactions with nitrites, nitrates, and Fe^{+2} at 1 μ M (Francisco, 2010; Klebanoff, 1989; Lu, 2008). At low concentrations, prooxidant interactions might outweigh the reduction of ROS from iron chelation, worsening the condition of the cells. In addition, the mixture of Fer-1 and DFO at 1 μ M had no synergic effect on ROS reduction ($p > 0.05$).

The results of this research can be used to establish that both Fer-1 and DFO are effective reducers of ROS that can be investigated for use for patients with AKI. At 10 μ M, DFO had a significant reduction of ROS at 1 and 24 hours ($p < 0.05$). At 1 μ M, Fer-1 had a significant ROS-reduction effect at 1 and 24 hours ($p < 0.05$). Fer-1 concentrations higher than 5 μ M should not be investigated for AKI treatments since there is no dose-dependent response after 5 μ M. Similarly, possible treatments for AKI should be aware of the negative consequences of DFO lower than 1 μ M and avoid low dose DFO treatments. Future researchers should try to identify the major mechanisms for DFO's prooxidant effect in HK-2 cells in order to understand conditions in which DFO treatments should be avoided in AKI patients.

Appendix A: P-Value Tables for Experiment 1

Table 1.

ANOVA Test P-Values between the Control and Hemoglobin & DFO Concentrations at 1 hour

	Hb+DFO 1 μ M	Hb+DFO 10 μ M	Hb+DFO 100 μ M	Hemoglobin
Control	0.0000179*	0.775	0.011*	0.000000757
Hemoglobin	0.872	0.0000218#	0.000000307#	N/A
Hb+DFO (10 μ M)	0.0000560			
Hb+DFO (100 μ M)	0.000000568	0.00200		

* p<0.05 with control, # p<0.05 with hemoglobin, **Overall: p=0.000000128 or 1.28e-08**

Table 2.

ANOVA Test P-Values between the Control and Hemoglobin & DFO Concentrations at 24 hours

	Hb+DFO 1 μ M	Hb+DFO 10 μ M	Hb+DFO 100 μ M	Hemoglobin
Control	0.0000000449*	0.00000207*	0.741	0.000000122*
Hemoglobin	0.409	0.011#	0.000000247#	N/A
Hb+DFO (10 μ M)	0.000815			
Hb+DFO (100 μ M)	0.0000000871	0.00000559		

* p<0.05 with control, # p<0.05 with hemoglobin. **Overall: p=0.0000000828 or 8.28e-09**

Table 3.

ANOVA Test P-Values between the Control and Hemoglobin & Fer-1 Concentrations at 1 hour

	Hb+Fer-1 1 μ M	Hb+ Fer-1 5 μ M	Hb+Fer-1 10 μ M	Hemoglobin
Control	0.424	0.00670*	0.028*	0.0000000456*
Hemoglobin	0.000000122 #	0.00000000346 #	0.00000000590 #	N/A
Hb+ Fer-1 (5 μ M)	0.000574			
Hb+Fer-1 (10 μ M)	0.00198	0.873		

* p<0.05 with control, # p<0.05 with hemoglobin, Hb in samples is at 5 μ M, Overall: p=0.0000000367 or 3.67e-09

Table 4.

12/16/20_24hrs ANOVA Test P-Values between the Control and Hemoglobin & Fer-1 Treatments

	Hb + Fer-1 1 μ M	Fer-1 5 μ M	Fer-1 10 μ M	Hemoglobin
Control	0.0000000777*	0.0000497*	0.00000504*	0.00000000811*
Hemoglobin	0.0146 #	0.00000126 #	0.00000930 #	N/A
Fer-1 5 μ M	0.0000438			
Fer-1 10 μ M	0.000728	0.176		

* p<0.05 with control, # p<0.05 with hemoglobin, Hb in samples is at 5 μ M, Overall: p=0.0000000116 or 1.16e-08

Appendix B: Raw Data of Relative Fluorescence in Experiment 1

Table 5.

12/16/20_1 hour Total ROS Relative Fluorescence Units for DFO & Fer-1 Treatments

	CTRL	Hb	Hb+ DFO (1μM)	Hb+ DFO (10μM)	Hb+ DFO (100μM)	Hb+ Fer-1 (1μM)	Hb+ Fer-1 (5μM)	Hb+ Fer-1 (10μM)
Well 1	3599	9380	9157	4741	313	3853	1519	2006
Well 2	3333	10543	10969	3615	187	3487	1171	1237
Well 3	2327	10409	8314	3213	160	4000	1239	1778
<i>Mean</i>	<i>3086</i>	<i>10111</i>	<i>9480</i>	<i>3856</i>	<i>220</i>	<i>3780</i>	<i>1310</i>	<i>1674</i>
<i>% of Hb</i>	<i>30.52</i>	<i>100</i>	<i>93.76</i>	<i>38.14</i>	<i>2.18</i>	<i>37.39</i>	<i>12.96</i>	<i>16.56</i>
<i>STDEV</i>	<i>670.92</i>	<i>636.31</i>	<i>1356.65</i>	<i>792.07</i>	<i>81.66</i>	<i>264.18</i>	<i>184.45</i>	<i>394.97</i>

Note: Hb=hemoglobin, All hemoglobin is at 5μM, STDEV=Standard Deviation, DFO=Deferoxamine, Fer-1=Ferostatin-1, the concentrations in the headings are for the DFO and the Fer-1. Also, all of the data is blank-corrected for increased accuracy.

Table 6.

12/16/20_24 hour Total ROS Relative Fluorescence Units for DFO & Fer-1 Treatments

	CTRL	Hb	Hb+ DFO (1μM)	Hb+ DFO (10μM)	Hb+ DFO (100μM)	Hb+ Fer-1 (1μM)	Hb+ Fer-1 (5μM)	Hb+ Fer-1 (10μM)
Well 1	7102	33696	38689	31713	8769	31425	19558	22067
Well 2	7005	37841	42711	27022	9670	28461	16978	19574
Well 3	5532	36702	36710	26284	7788	31360	18651	23766
<i>Mean</i>	<i>6547</i>	<i>36080</i>	<i>39370</i>	<i>28340</i>	<i>8743</i>	<i>30416</i>	<i>18396</i>	<i>21803</i>
<i>% of Hb</i>	<i>18.15</i>	<i>100</i>	<i>109.12</i>	<i>78.55</i>	<i>24.23</i>	<i>84.30</i>	<i>50.99</i>	<i>60.43</i>
<i>STDEV</i>	<i>879.78</i>	<i>2141.43</i>	<i>3057.91</i>	<i>2944.60</i>	<i>941.28</i>	<i>1692.81</i>	<i>1308.82</i>	<i>2108.50</i>

Note: Hb=hemoglobin, All hemoglobin is at 5μM, STDEV=Standard Deviation, DFO=Deferoxamine, Fer-1=Ferostatin-1, the concentrations in the headings are for the DFO and the Fer-1. Also, all of the data is blank-corrected for increased accuracy.

Appendix C: Experiment 2 Results

Table 7.

ANOVA Test P-Values between the Control and Hemoglobin & Experimental Groups at 24 hours

	Hb+DFO (1 μ M)	Hb+Fer-1 (1 μ M)	Hb+DFO+Fer-1 (1 μ M)!	Hb
Control	0.00000129*	0.000192*	0.000136*	0.0000510*
Hb	0.0121#	0.743	0.887	
Hb+Fer-1 (1 μ M)	0.00208		0.998	
Hb+DFO+Fer-1 (1 μ M)!	0.00319			

* p<0.05 with control, # p<0.05 with hemoglobin. Hb=Hemoglobin and all hemoglobin was treated at 5 μ M. !=Both DFO and Fer-1 were treated at 1 μ M.

Table 8.

1/13/21 24 hour Total ROS Relative Fluorescence Units for Treated Groups

	CTRL	Hb	Hb+DFO (1 μ M)	Hb+ Fer-1 (1 μ M)	Hb+DFO+Fer-1 (1 μ M)!	Hb+Fer-1 (5 μ M)	Hb+DFO (10 μ M)
Well 1	8546	27158	28671	21529	21738	13593	21134
Well 2	10206	24149	36359	24595	25790	16247	22223
Well 3	9785	24604	34272	23031	23335	15218	20747
Well 4					22289		
Well 5					22437		
Well 6					27059		
Mean	9513	25304	33101	23052	23775	15020	21368
STDEV	862.94	1621.93	3975.60	1533.10	2153.43	1338.11	765.32

Note: Hb=hemoglobin, All hemoglobin is at 5 μ M, STDEV=Standard Deviation, DFO=Deferoxamine, Fer-1=Ferostatin-1, the concentrations in the headings are for the DFO and the Fer-1. Also, all of the data is blank-corrected for increased accuracy.

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