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Deferoxamine produces nitric oxide under ferricyanide oxidation, blood incubation, and UV-irradiation

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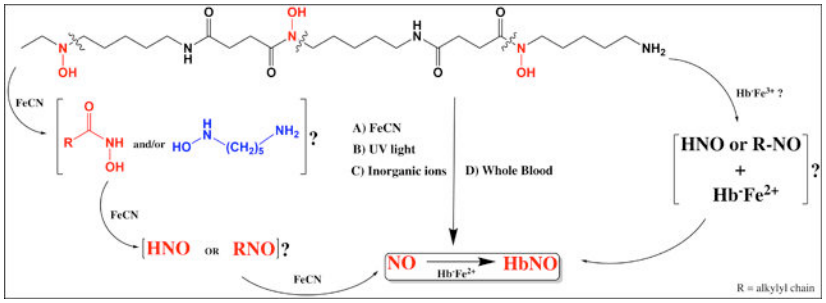
Abstract

Deferoxamine (DFO), an iron chelator, is used therapeutically for the removal of excess iron in multiple clinical conditions such as beta thalassemia and intracerebral hemorrhage. DFO is also used as an iron chelator and hypoxia-mimetic agent in in vivo and in vitro basic research. Here we unexpectedly discover DFO to be a nitric oxide (NO) precursor in experiments where it was intended to act as an iron chelator. Production of NO from aqueous solutions of DFO was directly observed by ozone-based chemiluminescence using a ferricyanide-based assay and was confirmed by electron paramagnetic resonance (EPR). DFO also produced NO following exposure to ultraviolet light, and its incubation with sheep adult and fetal blood resulted in considerable formation of iron nitrosyl hemoglobin, as confirmed by both visible spectroscopy and EPR. These results suggest that experiments using DFO can be confounded by concomitant production of NO, and offer new insight into some of DFO's unexplained clinical side effects such as hypotension.

Graphical Abstract



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Keywords

deferoxamine; nitric oxide; iron nitrosyl hemoglobin; ferricyanide

Introduction

Deferoxamine (DFO) is a widely used low molecular weight, high affinity iron chelating compound (siderophore) that is naturally produced by the bacteria *Streptomyces pilosus (Günter, Toupet, and Schupp 1993)*. Due to its iron chelating properties, whereby it specifically binds with Fe^{3+} (Richardson, Ponka, and Baker 1994), DFO has long been used clinically to remove excess iron, thus limiting iron toxicity in conditions such as hemochromatosis (Tenenbein 1996; Gumber et al. 2013) or secondary to transfusion therapy, especially in beta thalassemia patients (Kontoghiorghe and Kontoghiorghes 2016). In addition to its FDA-approved clinical use, DFO is also currently being investigated as a treatment for intracerebral hemorrhage in multiple ongoing Phase 2 clinical trials (Selim et al. 2011; Yeatts et al. 2013), after animal studies demonstrating its efficacy (Qing et al. 2009; Guo et al. 2019). DFO is also used experimentally as an iron chelator to probe cellular iron catabolism (Kim et al. 2016; Dayani et al. 2004), and as a hypoxia-mimetic agent due to its ability to induce HIF-1α overexpression (Bensaid et al. 2019; Lang et al. 2019), both in cell culture or tissue-based assays and in animal studies. During our recent experiments in which we were using DFO to decrease iron levels in cultured placental explants we serendipitously discovered that DFO produces nitric oxide (NO) under specific physico-chemical and biological conditions. This property of DFO does not appear to have been previously reported and may be an important consideration in its widespread clinical and investigational use.

DFO is a disubstituted tri-hydroxamic acid. Chemically, DFO has been considered quite stable under physiological and pathophysiological conditions. However, acetohydroxamic acid, hydroxyurea, Vorinostat and Belinostat, chemicals with structures similar to DFO (Figure 1), have all been thought to produce NO. NO and nitroxyl (HNO) release from unsubstituted mono-hydroxamic acids such acetohydroxamic acid (A. Samuni and Goldstein 2011; Yadav et al. 2014), and the anticancer drugs suberoylanilide hydroxamic acids (SAHA, Vorinostat) and Belinostat has been previously reported, especially under oxidative conditions (Kenny et al. 2019; Y. Samuni et al. 2014, 2009). Hydroxyurea has also been well documented in *in vitro* and *in vivo* animal and human studies to be capable of releasing NO and HNO, which may partly contribute to its use as treatment for cancer and sickle-cell disease (King 2005)(McGann and Ware 2015). Hydroxyurea rapidly increases concentrations of plasma nitrite as well as iron nitrosyl hemoglobin (HbNO), both of which are products of NO metabolism, in rats and human patients (J. Huang, Hadimani, et al. 2002; Jiang et al. 1997; Gladwin et al. 2002). Mechanistic studies with hydroxyurea or acetohydroxamic acid suggest that, under oxidative conditions such as in the presence of ferric heme proteins and hydrogen peroxide, hydroxyurea is oxidized to a nitroxide radical or a C-nitroso intermediate, which can in turn be oxidized to HNO and then NO (A. Samuni and Goldstein 2011; King 2004; Maimon, Samuni, and Goldstein 2018). These prior mechanistic studies also demonstrated that the HNO or NO could only be generated from

an unsubstituted acylhydroxylamine (–NHOH) group, since hydroxyurea analogues with an alkyl group instead of a hydrogen atom attached to the hydroxamate nitrogen failed to yield any NO (King 2004; J. Huang, Hadimani, et al. 2002; J. Huang et al. 2003). These results would make it difficult to predict that a disubstituted tri-hydroxamic acid such as DFO could generate NO and its subsequent bioactive derivatives. These include nitrate, nitrite, nitrosothiols, and iron nitrosyl complexes, collectively referred to as NOx.

In the current study, we report that DFO produces NO under ferricyanide oxidation, reaction with different buffers or buffer constituents, exposure to ultraviolet radiation, and reaction with sheep blood, as measured by ozone-based chemiluminescence and confirmed by visible spectroscopy and electron paramagnetic resonance (EPR). These findings raise the possibility that NO species (NOx) are increased in clinical conditions in which DFO is given and may have contributed to beneficial or deleterious side effects. The production of NOx by DFO could also be a confounding variable in many of the in vitro and in vivo experiments using DFO, especially when coupled with NO-based assays such as ones we (Mukosera et al. 2018) and others frequently use (Meczynska et al. 2008; Adgent et al. 2012; Sahni, Hickok, and Thomas 2018; Toledo et al. 2008; Bosworth et al. 2009). We therefore present our findings about the production of NO from DFO.

Materials and Methods

Ethical Approval.

Human placentas were collected at Loma Linda University Medical Center for studies of the metabolism of exogenous NOx in human placental homogenates under a protocol that was preapproved by the Loma Linda University Institutional Review Board (IRB #5180266). Animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University (IACUC# #8170004) and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chemical reagents and buffers.

Deferoxamine mesylate, potassium ferricyanide (FeCN) and HEPES were obtained from Sigma (St. Louis, MO). Phosphate buffered saline (PBS, pH = 7.4) was obtained from Corning (Corning, NY), and 1X Ca^{2+}/Mg^{2+}-free Hank's buffered salt solution (HBSS, pH = 7.4) by Gibco™ was purchased from ThermoFischer Scientific (Waltham, MA). Krebs-Ringer bicarbonate buffer (KRB, pH = 7.4) was prepared in the laboratory. Fetal bovine serum (FBS), L-Glutamine and penicillin/streptomycin were all obtained from Sigma (St. Louis, MO).

Human Placental Explant Culture.

All tissue culture experiments were performed in Iscove's Modified Dulbecco's Medium (IMDM) by Gibco™ obtained from ThermoFischer Scientific (Waltham, MA). Complete media was made by adding 10% FBS, 2 mM L-Glutamine, and 1% penicillin/streptomycin to the media. Villous explants (5 to 15 mg per explant) were sampled from the placental tissue between the chorionic and basal plates of placentas collected within 60 minutes following birth from women without pregnancy complications. Villous explants (3 per well

in a 12 well plate) were then cultured in 1.5 mL IMDM with and without 100 μM DFO for 6 hours, as a control for a study in which DFO was being used to scavenge iron in order to attenuate iron nitrosyl complex formation in the presence of exogenous NO adducts. Explant cultures were kept at 37 °C in a humidified incubator with 21% O_2 and 5 % CO_2. After 6 hours, placental explants were rinsed twice with 1 mL PBS then dried by blotting on clean paper. Explants were then homogenized in PBS buffer (8 μL per mg-tissue) and centrifuged for 15 minutes at 1,200 x g, after which the supernatant was collected and assayed for NOx species.

Adult and Fetal Sheep Blood and Plasma Collection.

Near-term, mixed western breed pregnant ewes were obtained from Nebeker Ranch (Lancaster, CA). After an overnight fast, blood was drawn from the jugular vein of unanesthetized ewes into heparinized syringes, and a portion of the blood was centrifuged within 30 minutes of collection at 1,000 x g for 15 minutes to isolate the plasma. The ewe was then anesthetized with intravenous ketamine (10 mg/kg) and midazolam (0.5 mg/kg) followed by intubation and ventilation with 3% isoflurane in oxygen. The uterus was accessed via a midline abdominal incision to enable collection of an umbilical blood sample and placental cotyledons. After further tissue sampling for other projects the ewe and fetus were euthanized with a lethal intravenous dose of Euthasol ®.

Ozone-based Chemiluminescence.

Nitric oxide was measured by an ozone-based chemiluminescence analyzer (Sievers NOA 280i, Boulder, CO). The NO analyzer is exclusively sensitive to the gaseous uncharged NO radical, which can be generated from a precursor molecule within a purge vessel containing a reductive or an oxidative reagent to facilitate the chemical release of NO from NO adducts. The NO analyzer does not detect other gaseous nitrogen oxides such as HNO, N_2O or NO_2, NO anions or NO cations. Triiodide (assay referred to as I_3^-), a reductive reagent in glacial acetic acid media, was used to determine the combined presence of all NOx species (nitrite, nitrosothiols, N-nitrosyls, and iron nitrosyl complexes, FeNO) except for nitrate. An oxidative reagent was prepared by adding 1 mL of 0.8 M potassium ferricyanide to 4 mL of pH 7.4 PBS buffer (assay referred to as PBS/FeCN), and the assay was used to selectively measure FeNO, e.g. HbNO, without nitrite and nitrosothiol interference (Mukosera et al. 2018). NOx concentrations were determined by quantification of peak areas plotted against a standard curve based on known nitrite standards prepared daily. Area under the curves were a linear function of concentration from 0.01 to 100 μM. The limit of detection was <0.01 μM.

In the course of this study, the PBS/FeCN assay, but not the I_3^- assay, was found to effectively release NO from DFO. All assays with PBS included 100 μl of 1-octanol as an anti-foam agent when biological samples were being measured.

I_3^- and PBS/FeCN reagents were each contained in separate purge vessels perfused by inert argon gas which carried the resultant NO to an NO analyzer. Samples of identical volumes were injected simultaneously into the reactant fluid of each purge vessel.

EPR Characterization.

Formation of iron nitrosyl hemoglobin (HbNO) in adult and fetal blood samples following incubation with 100 μM DFO for 24 hours was confirmed by EPR. EPR spectra of DFO-treated blood samples were measured at 150 K using a Bruker X-Band EMX Plus EPR spectrometer with a cavity of high sensitivity as previously described (Liu et al. 2018). The EPR was set to a microwave power of 20°CmW, microwave frequency of 9.34°CGHz, attenuator of 10°CdB, modulation amplitude of 1°CG, modulation frequency of 100°CkHz, time constant of 20.48 msec, conversion time of 81.92 msec, harmonic of 1, and number of scans of 2. $(MGD)_2Fe^{2+}$ (10°Cmmol/L MGD and 0.5°Cmmol/L Fe^{2+}) was prepared freshly in a glove box by dissolving $FeCl_2$ powder in Argon deoxygenated MDG solution. $(MGD)_2FeNO$ complex was measured at room temperature(Liu et al. 2019).

Probing NOx from reaction of DFO and FeCN.

A tandem purge vessel configuration (Fig 5A) was used to verify the production of gaseous NOx from the reaction of DFO and FeCN. By reacting DFO with FeCN in the first purge vessel and then directing the carrier argon gas into the second purge vessel which contained $(MGD)_2Fe$, the gaseous NOx produced in the first vessel could be captured by $(MGD)_2Fe$ in the second vessel while avoiding oxidation of $(MGD)_2Fe$ by the FeCN.

To test the possibility that DFO spontaneously generates HNO, which could then be oxidized by FeCN into the chemiluminescence-detectable NO, we designed four protocols (Fig 5E) with the use of different combinations of reagents in the tandem purge vessels.

NO production from DFO following UV-irradiation.

DFO has been previously reported to form the nitroxide radical under oxidative conditions (Morehouse, Flitter, and Mason 1987; Davies et al. 1987; Hinojosa et al. 1989). A few studies have shown that DFO at high concentrations (> 1 mM) can form the nitroxide radical, as a product of one-electron oxidation by the hydroxyl radical, which can be generated through photolysis of a dilute hydrogen peroxide solution under UV light (Morehouse, Flitter, and Mason 1987; Pieper, Gross, and Kalyanaraman 1990). We therefore determined whether nitrite formation could be observed from DFO samples under UV light with or without H_2O_2. Solutions of DFO (100 μM final concentration) in PBS, sheep plasma and sheep whole blood were then placed in an uncovered six-well plate, and exposed to UV light for two hours in a biosafety hood. Samples were collected at 30 minutes and 22 hours post the UV light exposure, and assayed for NOx by triiodide-based chemiluminescence.

Statistical Analysis.

Results are expressed as mean ± SD with p-values ≤ 0.05 considered statistically significant. All analyses were carried out using Graphpad Prism 8.0 software (Graphpad Software, La Jolla, CA).

Results

Unexpected Discovery of NO production by DFO in PBS/FeCN assay.

In the course of our experiments in which DFO was used to chelate iron in cultured placental explants we serendipitously discovered that DFO generates NO following ferricyanide oxidation. As shown in Figure 2A, supernatant samples from placental explants treated with 100 μM DFO released NO (1.0 ± 0.3 μM, p = 0.0003 vs control) upon injection into PBS/FeCN, but not in I_3^- (0.29 ± 0.05 μM, p = 0.99 vs. control). Injection of the DFO-treated medium, which had been in contact with the placental explants, into I_3^- (Figure 2A), and into PBS/FeCN (Figure 2B), similarly generated NO in PBS/FeCN (NO = 17 μM) but not in I_3^- (NOx = 0.3 μM). To test whether the placental tissue was needed for the observed NO-generation, fresh solutions of 100 μM DFO in deionized water and placenta-free cell media were prepared and injected into I_3^- (Figure 2D) and PBS/FeCN (Figure 2E) at both room temperature and after 20 minutes at 37 °C. NO production (> 20 μM) was once again observed in PBS/FeCN, but not in I_3^- (< 0.1 μM), for both the deionized water and cell media DFO solutions at both room temperature and 37 °C (Figure 2D, E). This finding shows that NO production is an inherent property of DFO under oxidative, but not reductive conditions, with no participation of biological tissue. All subsequent experiments were performed without inclusion of the placental tissue.

Characterization of NO formation from DFO in PBS/FeCN.

The dependency of NO production on DFO concentration was determined by injecting DFO, freshly made with deionized water or cell media solutions (1, 10, 50 and 100 μM), into a purge vessel with PBS/FeCN connected to the chemiluminescence NO analyzer. As shown in Figure 3A, the amount of NO generated from DFO by PBS/FeCN increases linearly with increasing DFO concentrations, although the absolute amount of generated NO was higher in deionized water (~ 50 μM NO per 100 μM DFO) vs. cell media (~ 20 μM NO per 100 μM DFO) as shown in Figure 3A, and the voltage tracings in Figure 3B vs. Figure 3C. Treatment of 100 μM DFO with a solution of FeCN in PBS for 20 minutes before injection into a purge vessel significantly attenuated the NO generation of the resulting solution upon injection to a purge vessel with PBS/FeCN (Figure 3D), consistent with the likelihood that DFO reacts directly with FeCN to generate NO in the vial, some of which was lost to ambient air, thus lowering the amount of NO injected into the purge vessel. NO release from DFO solutions (both deionized water and cell media solutions) in a purge vessel with PBS/FeCN was completely eliminated upon DFO pre-treatment with an aerobically prepared $FeSO_4$ (100 μM) solution (Figure 3E), suggesting that the iron-bound DFO might lose its NO-releasing property. Production of NO from DFO in PBS/FeCN was not affected by pre-treatment of 10 μM DFO with either 100 μM H_2O_2 or 100 μM cPTIO (Figure 3F). H_2O_2 is a pro-oxidant, which we expected to result in increased NO generation from DFO upon pretreatment and thus lower the signal upon injection into PBS/FeCN. The absence of an effect on NO generation from DFO in PBS/FeCN by cPTIO, an NO-scavenger, suggests that the NO generation does not occur spontaneously before injection into the purge vessel.

NOx formation from DFO solutions incubated with FeCN.

To confirm that DFO reacts directly with FeCN resulting in the formation of NO, we treated 10 μM DFO solutions in PBS or deionized water with 80 mM FeCN (in deionized water) in a closed vial. We then used triiodide-based chemiluminescence to detect NOx formation from the oxidation of any NO generated from the DFO under FeCN in the presence or absence of various ion species. Since the I_3^- assay does not detect nitrate, and there were no thiols or heme iron centers in the buffers for nitrosothiol and iron nitrosyl complex formation respectively, we regarded any formed NOx as nitrite (which does not exclude nitrate formation). The identity of the NOx as was confirmed by injecting the sample in a purge vessel containing ascorbic acid in a pH = 2.0 HCl/KCl buffer, a nitrite-selective purge vessel reagent, which yielded similar results to those obtained in triiodide (data not shown). Consistent with DFO oxidation to NO, as shown in Figures 4A and 4B, nitrite formation was observed upon injection of the FeCN-treated DFO solutions that were prepared in PBS, and the amount of nitrite increased linearly with time, at a rate of 0.15 μM nitrite per minute. The reaction was stopped at 15 minutes, a time point at which 2.0 μM nitrite (20 % of the initial DFO concentration) had been produced from DFO. In contrast, no nitrite formation was observed from FeCN-treated DFO solutions that were prepared in deionized water (Figure 4A). This suggested that NO generation from DFO by FeCN requires the presence of an ionic component that is in PBS. We then made 10 μM DFO solutions in PBS, or its composite ions (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO4) followed by addition of 80 mM FeCN in deionized water. Nitrite formation was determined following incubation for 10 minutes at room temperature. As shown in Figure 4C, nitrite formation is only observed in the DFO solutions in PBS or 10 mM Na_2HPO4 following FeCN incubation, suggesting that the $HPO4^{2-}$ ion facilitates the oxidation of DFO, or a metabolite of DFO, by FeCN.

We then checked for similar nitrite formation in other commonly used buffers, such as KRB, HBSS, and HEPES. Nitrite formation was indeed observed in all the tested buffers (Figure 4D) suggesting they too facilitated oxidation of DFO (or a DFO metabolite) by 10 mM FeCN to yield NO, that is then oxidized to nitrite. We then determined whether some of the inorganic salts (HCO_3^-, CO_3^{2-}, HPO_4^{2-} and PO_4^{3-}) commonly used in buffers or other preparations were responsible. As shown in Figure 4E, only the carbonate ion directly (without FeCN) reacted with DFO to yield nitrite; it provided ~ 10 μM nitrite within one minute of incubating 100 μM DFO in 10 mM CO_3^{2-} solution, suggesting a spontaneous reaction. Upon addition of FeCN, nitrite formation from 100 μM DFO was observed in all ionic solutions of DFO as shown in Figure 4F, but was highest in CO_3^{2-} (20 μM nitrite at peak) and lowest in HCO_3^- (7 μM nitrite at peak).

Probing possible HNO intermediacy.

The production of NO by DFO following oxidation by FeCN was independently confirmed by EPR and further validated by chemiluminescence using two purge vessels connected in series, a setup that is illustrated in Figure 5A. For EPR, the reaction product of 100 μM DFO and 100 mM FeCN in purge vessel **I** was purged by argon into purge vessel **II** containing $Fe(MGD)_2$, an NO spin-trapping molecule. The result was an EPR signal characteristic of the $Fe(MGD)_2$-NO complex, as shown in Figure 5B. It is worth noting,

however, that $Fe(MGD)_2$ has also been shown to equally react with HNO, yielding the same characteristic $Fe(MGD)_2$-NO complex (Komarov et al. 2000). However, since ozone-based chemiluminescence is selective for NO and does not directly detect gaseous HNO, and since the excess FeCN in purge vessel **I** would equally oxidize any HNO to NO, we concluded that NO is the end product of DFO oxidation by FeCN.

Since both HNO and DFO can be oxidized under FeCN to produce NO, it is possible that DFO spontaneously generates HNO, which is subsequently oxidized to NO by FeCN, thus making DFO a previously characterized HNO donor. It is also likely that the oxidation of DFO is a multistep process, which ultimately results in the i*n situ* formation of HNO, which is subsequently oxidized to NO. To determine the spontaneous formation of HNO from DFO we utilized the tandem purge vessel setup (Figure 5A), with a chemiluminescence NO-analyzer connected to purge vessel **II**. Purge vessel **I** contained PBS while purge vessel **II** contained FeCN, so as to uncouple any spontaneous HNO generation from FeCN oxidation. Angeli's salt, an HNO donor, was used as a positive control for spontaneous HNO generation. When 100 μM of Angeli's salt (in 1 to 10 mM NaOH) was added to purge vessel **I** with PBS, in series with purge vessel **II** containing FeCN, a broad NO signal (50 μM upon integration), was observed (Figure 5C). In contrast, Angeli's salt added to PBS without FeCN does not yield any NO signal (data not shown). The experiment was then repeated for DFO as shown in Figure 5D. As shown in configurations 1–3 of Figure 5D, no NO was observed when DFO was injected in a purge vessel without FeCN. As shown in configuration 4 of Figure 5D, NO is only generated when DFO is injected directly to FeCN. This strongly suggests that DFO does not generate HNO spontaneously, but does not preclude the possibility that DFO, or any intermediary, is first oxidized to HNO in the presence of FeCN, which is then further oxidized to NO by excess FeCN.

Based on the structure of DFO it seems likely that the oxidation of DFO into NO proceeds through either a hydroxamic acid (e.g. acetohydroxamic acid) and/or a hydroxylamine (e.g. 5-(hydroxyamino)-pent-1-amine) intermediate, depending on the cleavage pattern, as illustrated in Figure 6A. We therefore determined the amount and rate of NO release from 10 μM solutions of Angeli's salt (HNO), DFO, acetohydroxamic acid (AHA), and hydroxylamine (NH_2OH). By comparing the rate of NO release, we can determine which of the possible intermediates have rates of NO release faster than DFO, and are thus more likely to be intermediates. As shown in Figure 6B and 6C, only Angeli's salt generates NO under acidified triiodide. AHA, DFO and NH_2OH do not release NO under acidified triiodide. On the other hand, Angeli's salt (HNO), AHA, DFO and NH_2OH all appear under PBS/FeCN (Figure 6D–G) with rates of NO release: HNO > AHA > DFO > NH_2OH (Table 1). Signals from DFO and NH_2OH are notably broader than those from HNO and AHA due to the former's slow rate of NO generation.

Angeli's salt is known to release an equimolar amount of both nitrite and nitroxyl (Paolocci et al. 2007); the calculated concentration (~ twice the Angeli's salt concentration) as well as the broad NO-release profile (different from nitrite) indicates that both nitrite and nitroxyl from Angeli's salt generate NO under triiodide. HNO, but not DFO, generates NO under triiodide, indicating DFO does not spontaneously generate HNO, consistent with results in Figure 5. However, based on results from Figure 6D–G and Table 1,

the quick generation of NO from HNO and AHA under FeCN vs. DFO strengthens the suggestion that DFO is probably oxidized to an acetohydroxamic acid derivative first, which then directly releases NO upon oxidation by FeCN, or is itself oxidized to HNO that is then quickly oxidized to NO by the excess FeCN. This suggestion is strongly supported by results from mass spectrometry with liquid chromatographic separation of DFO fragments following DFO's reaction with FeCN as described in Supplementary Figure 1 and illustrated in Supplementary Figure 2. Since hydroxylamine generates NO much slower than DFO, hydroxylamine derivatives are unlikely to be an intermediate in the generation of NO by DFO. However, mass spectrometry results also suggested a possible C-nitrosopentamine intermediate (Supplementary Figures 1 and 2), which we did not confirm by chemiluminescence.

NO production from DFO under UV-Light.

DFO has been previously reported to form the nitroxide radical under UV light in the presence of H_2O_2 (Morehouse, Flitter, and Mason 1987; Pieper, Gross, and Kalyanaraman 1990), although we are not aware of any reports on whether DFO also generates NO under the same conditions. As shown in Figure 7, exposure of DFO to UV light for 2 hours results in significant NOx formation in PBS (Figure 7A), sheep plasma (Figure 7B) and sheep whole blood (Figure 7C) both at 30 mins and at 24 hours post UV-exposure. NOx results are summarized in Table 2. NOx formation by DFO following UV exposure was not affected by the presence of H_2O_2 (100 μM and 1 mM), suggesting that nitroxide formation is not a significant part of the NOx generation observed under UV.

Furthermore, the whole blood samples yielded peaks characteristic of HbNO formation (data not shown) suggesting that DFO generates NO under UV, which is oxidized to nitrite (and possibly nitrate: not assayed for) in PBS and plasma samples, but is trapped by Hb in blood to yield HbNO. Alternatively, DFO, following activation by UV-light, reacts directly with Hb species in whole blood resulting in the observed HbNO species. We did not assay for nitrate formation in the PBS, plasma or whole blood samples at either 30 mins or 24 hours, though we expect nitrate levels to be even higher than the nitrite or HbNO levels that we detected in whole blood due to the reaction of any formed NO with oxyhemoglobin. Our results therefore estimate that a minimum of 1 to 5 % DFO is converted to NO in 24 hours following exposure to UV light in the absence of any FeCN.

HbNO formation from DFO in sheep blood.

To determine whether NO formation is a pathway in the metabolism of DFO in blood we incubated DFO in sheep adult or fetal blood. Both adult and fetal blood were tested since DFO is often employed by pregnant women as well (Piccioni et al. 2020; Singer and Vichinsky 1999), although there are concerns about its safety in this clinical group. A concentration of 100 μM DFO was prepared in whole blood, or blood lysed by three freeze-thaw cycles and incubated for 24 hours at 37 °C, at the end of which samples were immediately assayed for NOx by triiodide-based chemiluminescence, or snap frozen for UV-Vis and EPR spectroscopy.

As shown in Figure 8A, incubation of 100 μM DFO with adult sheep blood resulted in mostly no NOx formation, or sometimes very little NOx formation, in adult whole blood (0.5 ± 1 μM NOx, $n = 8$). Significantly more NOx formation was observed in adult lysed blood (12 ± 9 μM NOx, $n = 8$). Interestingly, fetal blood samples provided more NOx formation from DFO, with whole blood yielding 72 ± 45 μM NOx ($n = 8$), and lysed blood yielding 92 ± 15 μM NOx ($n = 8$). The reaction of DFO with blood, however, demonstrated much variability as evident in the standard deviation values, with a few experiments yielding no NOx.

As shown in Figure 8B, and similar to observations of DFO in whole blood under UV light, the voltage tracing of the NOx (broad peaks) obtained after 24 hour incubation with sheep whole and lysed blood were characteristic of HbNO instead of nitrite/nitrosothiols. The presence of HbNO in the blood samples was confirmed by visible absorption spectroscopy followed by the deconvolution of the spectrum by multiple linear regression analysis using standard spectra of the individual components (deoxyheme, oxyheme, iron-nitrosyl-heme, and metheme) (Z. Huang et al. 2005). Representative visible absorption spectra of adult and fetal blood samples (whole and lysed blood) after incubation with DFO for 24 hours are shown in Figure 8C. Spectral deconvolution confirmed the presence of mainly HbNO and metheme at the end of the 24 hour incubation period.

The presence of HbNO in the adult and fetal sheep blood samples was further validated by EPR, which yielded the same trends obtained by triiodide-based chemiluminescence. HbNO has a unique EPR triplet signal around the g = 2.0 region of an EPR spectrum. As shown in Figure 8D, no HbNO characteristic peak is observed for DFO in adult whole blood, while a small HbNO peak is observed in lysed adult whole blood. Figure 8E shows the HbNO peak observed from DFO in fetal whole blood, while Figure 8F shows the HbNO peak observed from DFO in fetal lysed whole blood. Consistent with the triiodide-based chemiluminescence results, the EPR-detected HbNO signal was stronger in fetal blood samples compared to the adult blood.

Discussion

We here report our unexpected discovery that deferoxamine, an iron chelator widely used in clinics as well as basic research, produces NO under specific conditions. We have shown that DFO is oxidized in PBS/FeCN solutions in a purge vessel to produce NO that is detected directly by chemiluminescence. NO is also produced in FeCN solutions in a closed vial and is subsequently oxidized to nitrite, which is then detected by I_3^- based chemiluminescence. Interestingly, I_3^-, a reducing agent, does not produce NO from DFO, further emphasizing the need for oxidative conditions to facilitate the generation of NO from DFO. Another interesting feature of this reaction is that it is facilitated by commonly used buffers such as PBS, HEPES, KRB and HBSS as well as by specific composite inorganic ions present in these buffers. The oxidation of DFO by FeCN to produce NO is not observed in pure deionized water, in the absence of any relevant ion. This suggests that the oxidation of DFO to NO has a high activation barrier, which is only lowered in the presence of the appropriate inorganic ions, either by lowering the oxidation potential of DFO, or by stabilizing the transition state of the reaction, or by another catalytic mechanism. Notably,

spontaneous nitrite formation is observed for DFO solutions in 10 mM CO_3^{2-} even without FeCN treatment, indicating that there could be alternative pathways, other than oxidation, in which NO is generated from DFO.

The NO is generated from DFO under oxidative conditions of PBS/FeCN. Other hydroxamic acids have also been reported to generate NO, at least partially, through the intermediacy of HNO (Maimon, Samuni, and Goldstein 2018; Y. Samuni et al. 2014). We therefore hypothesized that DFO acts as a donor of HNO, which is slowly released in PBS or inorganic ions, and then oxidized to NO in the presence of FeCN. We then used two in-series purge vessels to uncouple the putative HNO formation from ferricyanide oxidation. We achieved this by adding DFO to a purge vessel with PBS, under a stream of argon gas that would carry any spontaneously generated HNO into the next purge vessel containing FeCN for oxidation and NO formation. Since we did not observe any NO formation when DFO was added to the PBS-containing purge vessel in series with a subsequent FeCN-containing purge vessel, it seems unlikely that the DFO spontaneously produces HNO. Comparison of DFO vs. HNO, AHA and NH_2OH in both FeCN and triiodide reagents by chemiluminescence revealed that HNO quickly generates NO under both FeCN and triiodide. In contrast, DFO, AHA or NH_2OH, do not generate any NO under triiodide, and have relatively slower NO generation under FeCN. This also suggests that DFO does not spontaneously generate HNO, since any spontaneously generated HNO would be detected by the acidified triiodide. A combination of LC/MS (Supplementary Fig 1) and chemiluminescent results, however, strongly suggest that DFO generates NO through an unsubstituted hydroxamic acid intermediate. This intermediate is oxidized to HNO, which itself is then oxidized to NO by FeCN. Alternatively, as suggested by LC/MS, DFO generates NO through a C-nitrosopentamine intermediate, which is then oxidized to NO by FeCN, similar to HNO. C-nitroso intermediates have been observed in the oxidation of other hydroxamic acids to NO (J. Huang, Sommers, et al. 2002; Maimon, Samuni, and Goldstein 2018). However, purely mechanistic studies regarding the production of NO from DFO still need to be performed to conclusively determine the intermediacy of HNO, and confirm the intermediacy of a C-nitroso compound.

We also demonstrated that DFO generates NO following incubation of DFO under UV-light for 2 hours, as determined as nitrite and HbNO formation in PBS/plasma samples or whole blood samples respectively. This result suggests that the formation of NO from DFO requires a homolytic fission step, which is the common bond dissociation pattern under UV light, yielding two radicals. It is likely that homolysis of DFO results in the formation of a radical intermediate that ultimately yields NO slowly since we only observe significant nitrite formation a couple of hours following UV exposure. Our results however suggest that the formation of NO from DFO is not dependent on formation of the nitroxide radical, previously detected in DFO under oxidative conditions including a system of UV light with H_2O_2 (Hinojosa et al. 1989; Pieper, Gross, and Kalyanaraman 1990; Morehouse, Flitter, and Mason 1987), since there is no difference in NO generation with or without H_2O_2 under UV light in pH = 7.4 PBS. Although only described preliminarily here, the observation that DFO generates NO under UV light could help in deciphering the molecular mechanism through which the disubstituted DFO molecule ultimately generates the NO radical, a process which

would require the breaking of at least two C-N bonds and the abstraction of a proton (-NOH).

We recently reported a PBS/FeCN based assay for the selective detection of iron nitrosyl complexes (FeNO) (Mukosera et al. 2018). It is worth noting that DFO will interfere with the detection of FeNO in this assay as well as other NO-based assays (Thomas et al. 2018). The capability of DFO to generate NO, in light of NO's capacity to alter cellular signaling, should also be considered in experiments employing DFO as an iron chelator or hypoxia-mimetic agent. For instance, a study by Martinez-Romero noted increases in total NOx (nitrate/nitrite and S-nitroso compounds) levels following 24 hours of 200 μM DFO exposure to murine fibroblasts (Martínez-Romero et al. 2008). The NOx increase was associated with accumulation of HIF-1α. Although their results were partially explained by induction of iNOS through a PARP-1 dependent mechanism, it is also possible that some of the observed NOx was generated by DFO, especially since they still observe increased NOx in cells with either pharmacologic inhibition or genetic ablation of PARP-1. Any NO generated from DFO in this and similar experiments could easily confound NO-dependent cellular signaling such as HIF-1α or NfκB expression. For experiments involving NO measurements or NO signaling, it would therefore be advisable to use alternative iron chelators such as pyridoxal isonicotinoyl hydrazone (PIH).

We also demonstrate that HNO, acetohydroxamic acid (and thus possibly many other hydroxamic acids), and hydroxylamine all generate NO following reaction with PBS/FeCN assay, and that HNO, but not acetohydroxamic acid and hydroxylamine, generates NO following reaction with triiodide. This raises the need for caution when working with these chemicals with the different reagents often used in conjunction with ozone-based chemiluminescence.

Finally, since DFO is already widely used clinically, we probed whether the production of NO from DFO would occur under biological conditions and at common therapeutic concentrations. Plasma concentrations of DFO are reported to reach between 80 and 130 μM following 3 minutes of intravenous injection (Selim 2009). We discovered by chemiluminescence, visible spectroscopy and EPR that the incubation of DFO with sheep blood results in the formation of HbNO over 24 hours, more efficiently in lysed compared to whole blood, and significantly more efficiently in fetal vs. adult sheep blood. While DFO is reported to have poor membrane permeability (Hamilton et al. 1994), it is frequently used as a chelator in iron overload due to hemolytic conditions such as hemolytic anemia (al-Rimawi et al. 1999), which are also associated with methemoglobinemia. Our results suggest potential HbNO formation from DFO in these clinical conditions, as well as in fetal or neonatal blood following pregnant or neonatal use. DFO is often employed during pregnancy (Piccioni et al. 2020; Singer and Vichinsky 1999), although there are concerns about its safety due to some teratogenic effects observed in animal studies. HbNO production from DFO, which we observe in fetal blood might be helpful in determining the maternal or neonatal conditions when DFO use might be helpful or when the risks outweigh the benefits.

The strong HbNO signal that we observe could possibly be due to the production of NO that was ultimately captured by deoxyhemoglobin resulting in HbNO formation. Alternatively, it is possible that the DFO molecule is directly oxidized by the Fe^{3+} center in methemoglobin abundant in the lysed blood as has been reported for nitroxyl or other hydroxamic acids (Bazylinski and Hollocher 1985; King 2005; J. Huang et al. 2003). The latter option is more likely since it explains the marked HbNO production that we observe even in the presence of significant oxyhemoglobin concentrations, which normally would rapidly oxidize any free NO to produce nitrate (Blood et al. 2009). Also, fetal hemoglobin is known to have stronger affinity for diatomic gaseous molecules such as O_2, CO, and possibly NO as well, which could explain why significantly higher HbNO is obtained with fetal compared to adult blood.

It is however worth noting that the reaction resulting formation of HbNO from DFO following in vitro incubation was found to be inconsistent resulting in very large variability between samples, with a few samples (especially adult blood samples) even yielding no signal at all. While we hope to fully characterize the conditions that affect this reaction such as determining the optimal oxygen saturation, it is still noteworthy that DFO indeed produces NOx species in both adult and fetal blood. These results set the stage for in vivo application of DFO in animals to determine whether this results in NO formation in both blood and organ samples (Jiang et al. 1997; Lockamy et al. 2003), as well as determining whether patients who routinely take DFO as medication have elevated plasma and whole blood NOx levels, as has been discovered for hydroxyurea (Gladwin et al. 2002; Glover et al. 1999). It is possible that the generation of NOx species from DFO in blood contributes to the known therapeutic effect of DFO as well as some of its well-characterized side effects such as hypotension (Selim et al. 2011; Westlin 1966). DFO has also been reported to increase NO production when used in malaria patients (Weiss et al. 1997), a phenomenon attributed to increased expression of NO synthases (Fritsche et al. 2001). Likewise, others have reported increased eNOS expression in DFO experimental groups (Ikeda et al. 2011). It is possible that those findings of increased NO production can be explained, at least in part, by the non-enzymatic generation of NO from DFO.

Conclusion

We have demonstrated by ozone-based chemiluminescence, EPR and visible spectroscopy that DFO generates NO, especially under oxidative conditions, but also in solution with inorganic ions such as carbonate, and under UV light at near neutral pH. We also presented our preliminary results showing the formation of HbNO from DFO incubated with sheep blood. While we did not decipher the mechanism through which NO is generated from DFO, our preliminary results suggest that this is likely through the intermediacy of a C-nitroso species, which can be oxidized to NO by either FeCN in solution or Fe^{3+} in blood. These results are of importance for experiments using DFO as an iron chelator both in vivo or in vitro, and also of clinical importance since DFO is widely used clinically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights: DFO produces NO

1. Deferoxamine (DFO), a commonly used iron chelator, releases nitric oxide (NO) under oxidative conditions as well as under UV light.
2. Iron nitrosyl hemoglobin (HbNO) is also significantly formed from DFO incubated with sheep blood
3. NO release from DFO could confound experiments using DFO, and offer new insight into some of DFO's unexplained clinical side effects such as hypotension

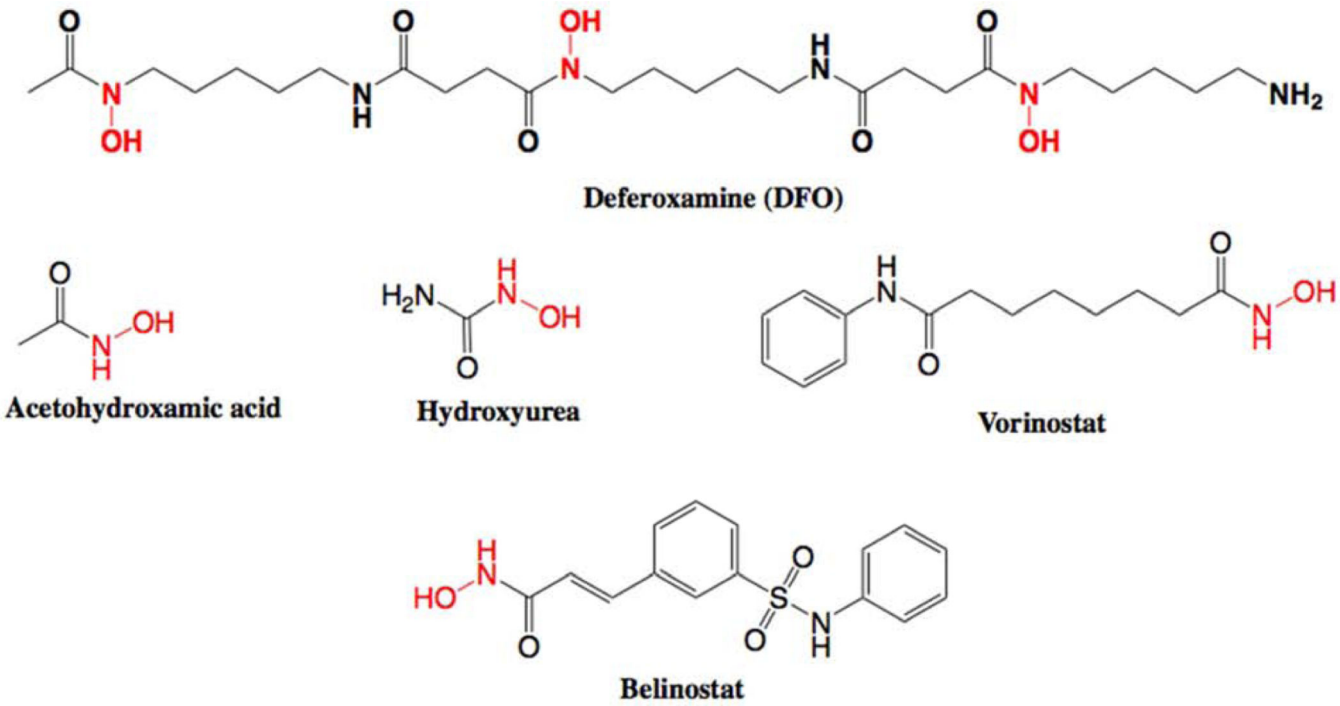


Figure 1.

Chemical structures of hydroxamic acids DFO, acetohydroxamic acid, hydroxyurea, vorinostat and Belinostat. NO generation has been previously reported for the latter four chemicals.

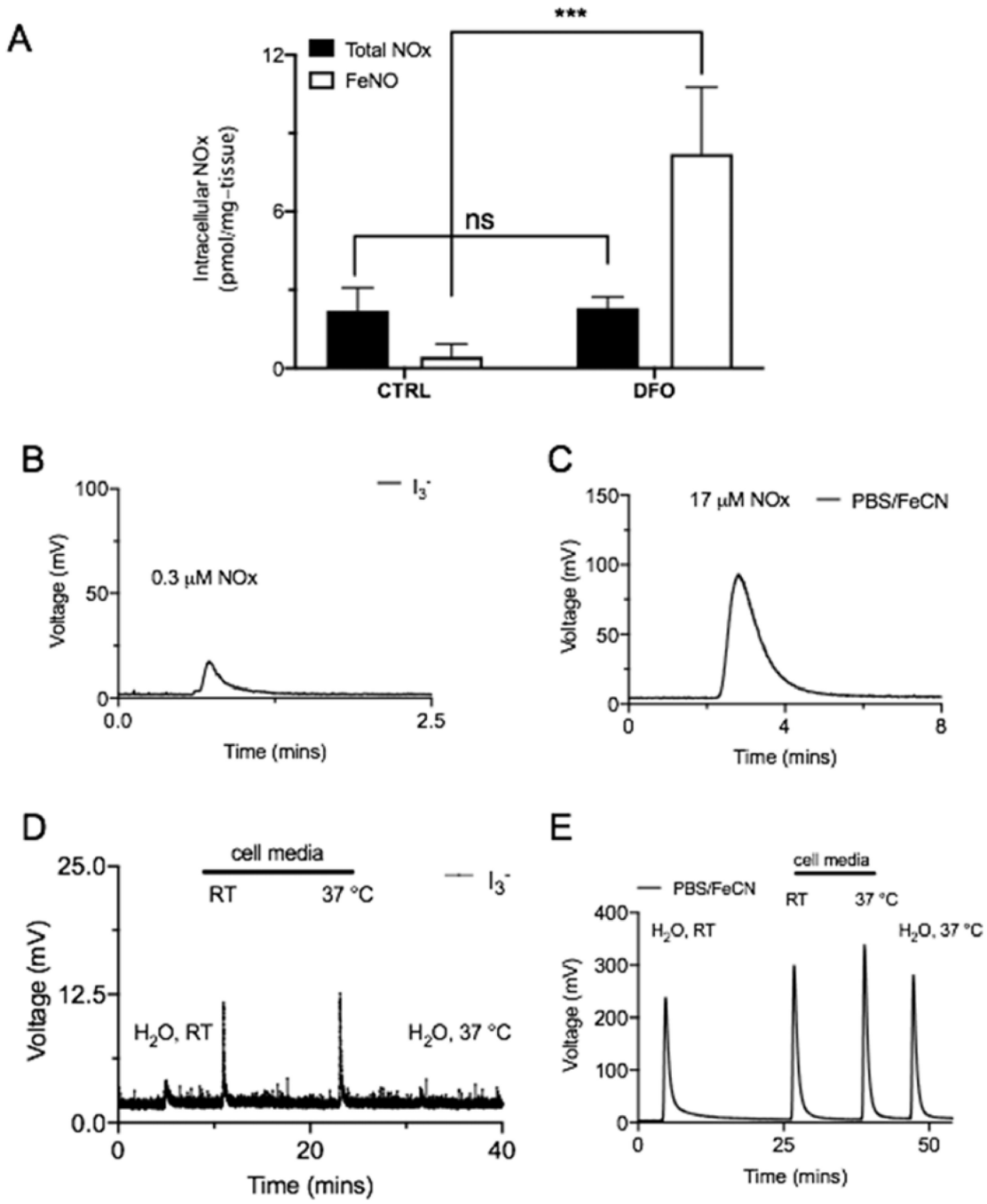


Figure 2.

Discovery of NOx production from DFO in PBS/FeCN assay. A) Chemiluminescent measurement of NOx in homogenates of placental explants incubated with DFO. Human placental explants were cultured in media containing 100 μM DFO for 6 hours, and homogenized in PBS buffer. The resulting supernatant was injected into a purge vessel containing triiodide (I_3^-) to measure total NOx concentrations, and another one containing PBS/FeCN reagents, which is normally used to detect iron nitrosyl (FeNO) species by chemiluminescence. DFO had no effect on the NO signal in I_3^-, but increased the NO

signal in the PBS/FeCN assay. NOx concentrations in tissue culture media containing 100 μM DFO after 6 hours of incubation with human placental explants were assayed by I_3^- (B) to yield only 0.3 μM NOx, and by PBS/FeCN (C) to yield 17 μM NO detected by chemiluminescence, suggesting that the NO production observed in PBS/FeCN is an inherent property of DFO, as opposed to metabolism of the DFO by the placental tissue. 100 μM DFO solutions in deionized water and tissue culture media were subsequently prepared and similarly injected into tri-iodide (D) or PBS/FeCN (E), where NO production was observed only in PBS/FeCN, demonstrating the NO production is an inherent DFO property that occurs via reaction with FeCN.

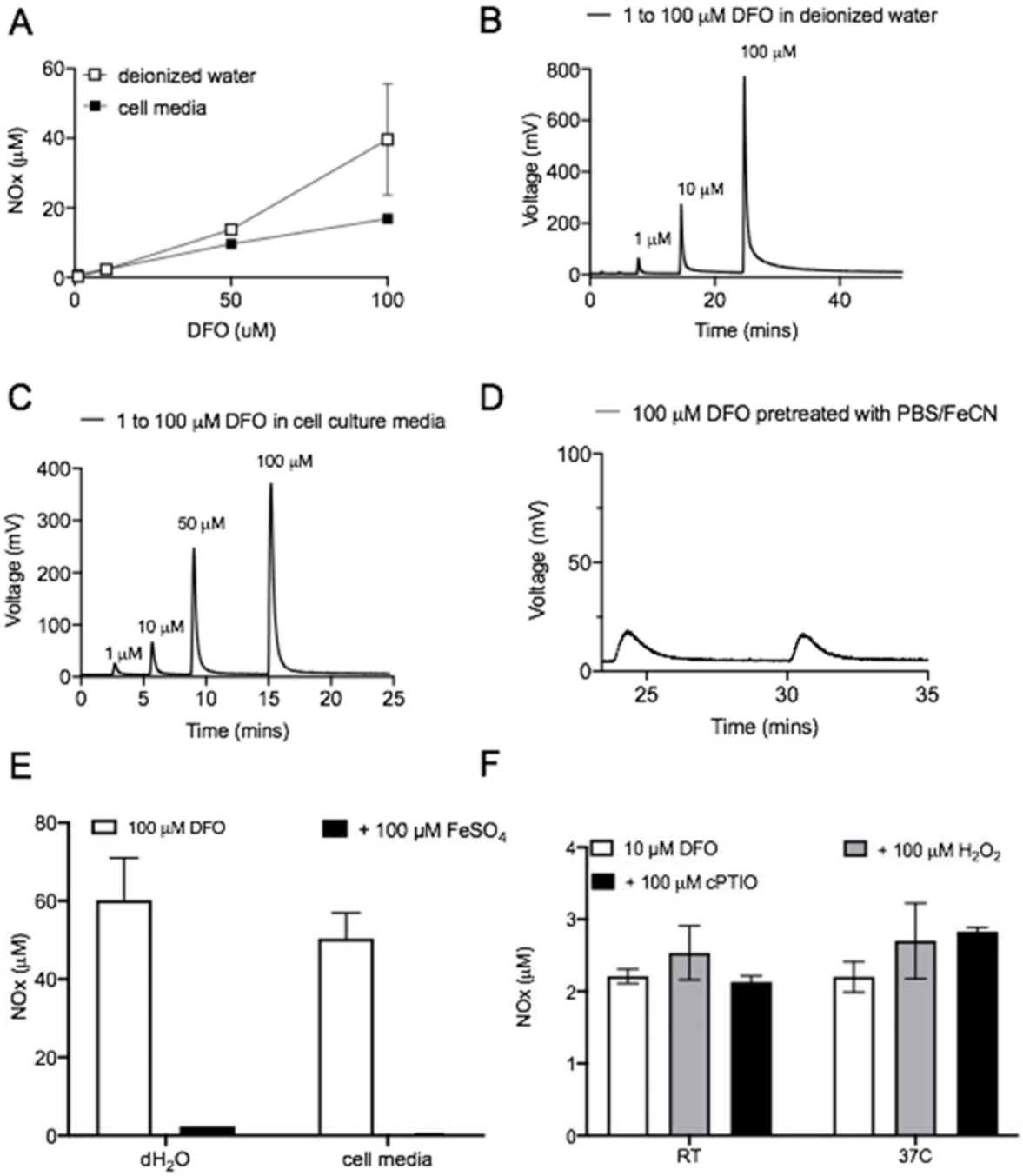


Figure 3.

NO generation by deionized water or cell culture media solutions of DFO upon injection to a purge vessel with PBS/FeCN as detected by chemiluminescence. A) Relationship between DFO concentration in deionized water or tissue culture media and the amount of NO generated from DFO following reaction in a purge vessel with PBS/FeCN. The voltage tracing for the NO generated from 1 to 100 μM DFO in deionized water (B) or tissue culture media (C) following reaction in a purge vessel with PBS/FeCN. D) Representative NO signal trace of two injections of 100 μM DFO in deionized water pre-treated by a 10 mM

FeCN solution for 20 minutes in PBS. Injections were into a purge vessel with PBS/FeCN. The NO signal is significantly diminished following the pre-treatment as compared with that shown in (B). Effect of pre-treating 100 μM DFO solutions in deionized water and tissue culture media with 100 μM of $FeSO_4$, which eliminates the DFO signal (E), and F) Effect of treating deionized water solutions of DFO (1o μM) with 100 μM H_2O_2 or cPTIO at either room temperature or 37 °C. Both H_2O_2 and cPTIO have a minimal to no effect on the amount of NO generated from DFO upon reaction with PBS/FeCN in a purge vessel. Measurements were performed in duplicates, and repeated on at least two different days.

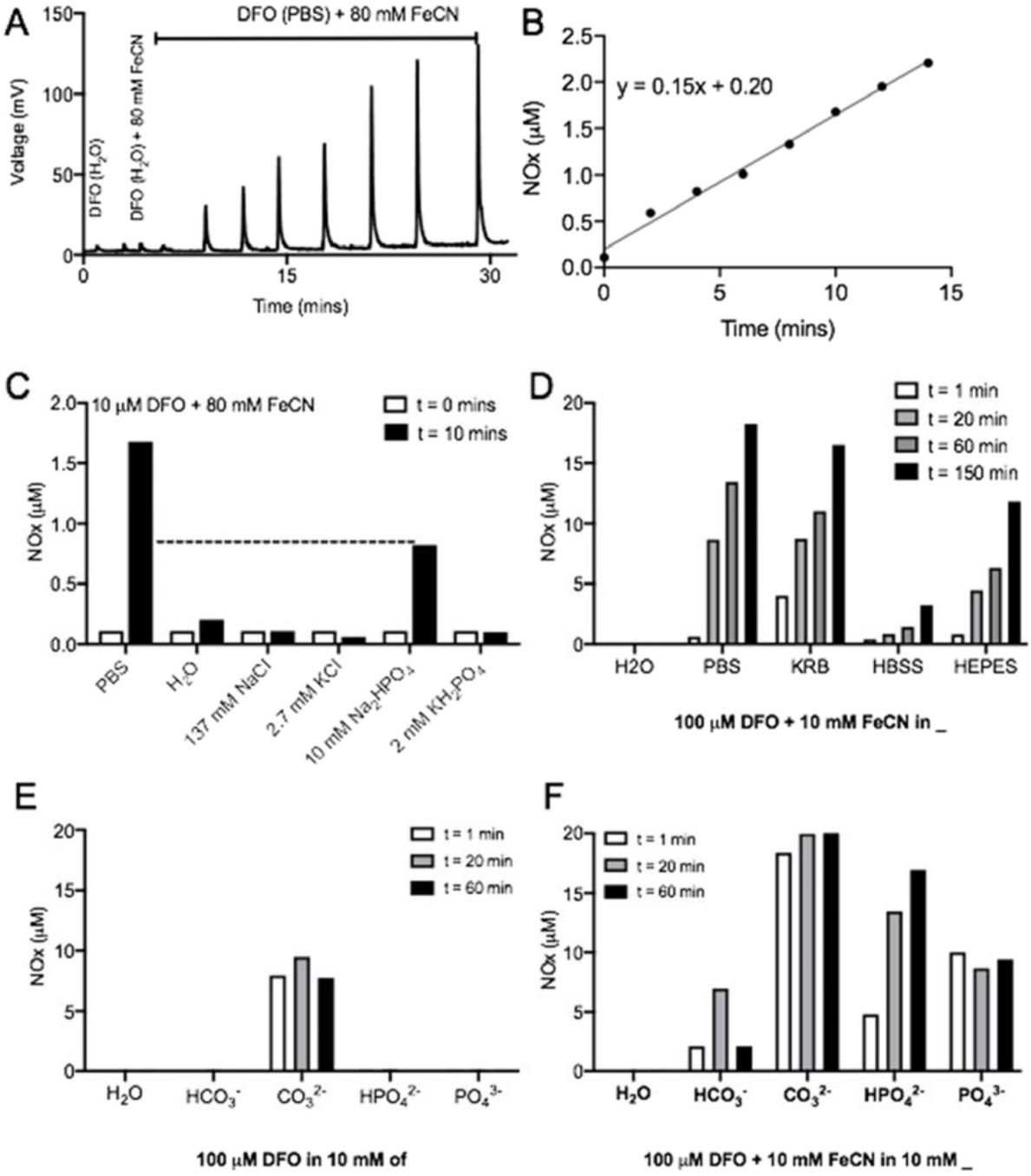
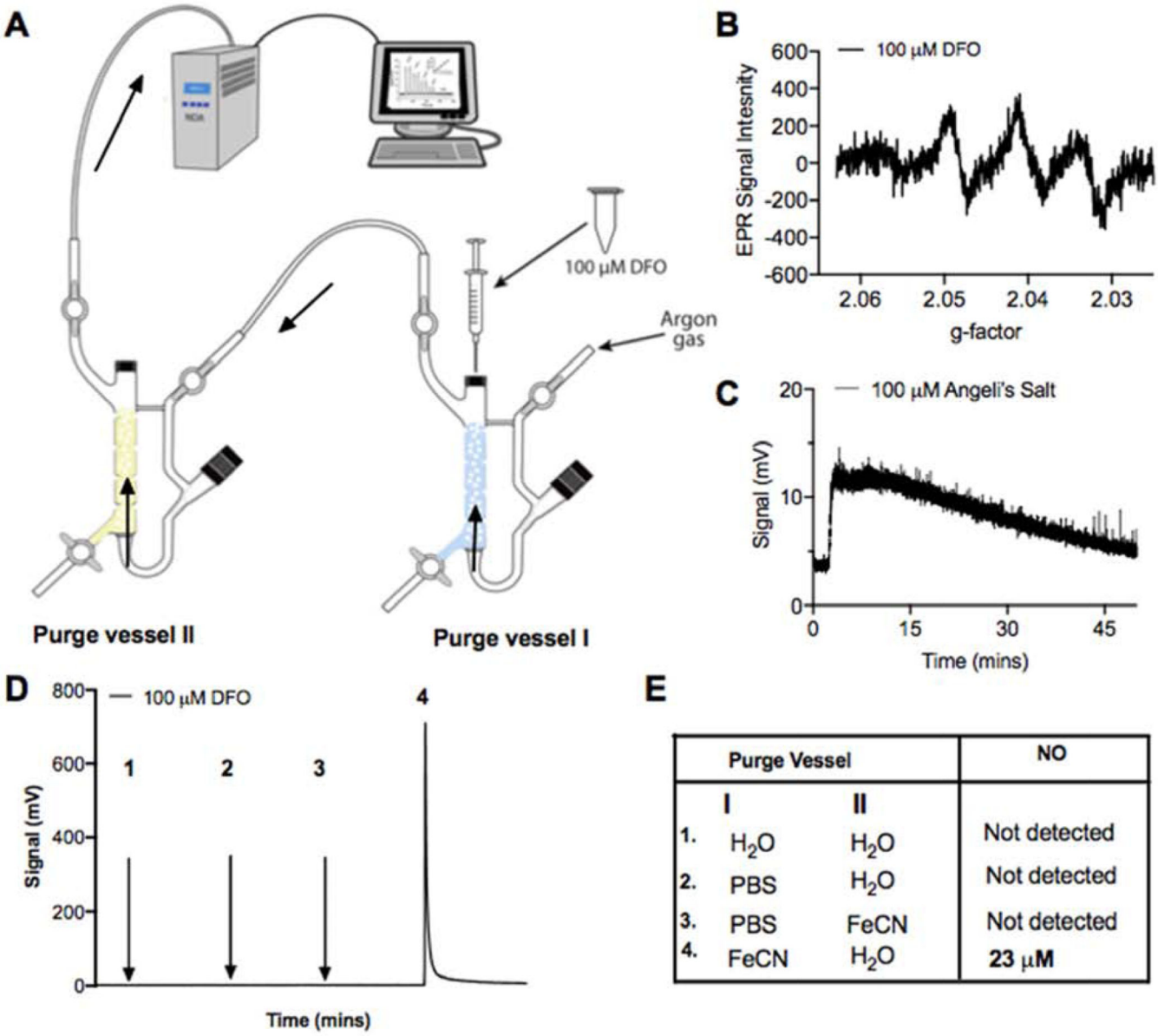


Figure 4.

The effect of buffer systems on nitrite formation from DFO solutions incubated with FeCN, followed by measurement with I_3^- reagent. Voltage tracing (A) and the corresponding calculated nitrite concentrations (B) upon incubation of 10 μM DFO solutions (made in PBS) with 80 mM FeCN solution (made in deionized water) for 15 minutes at room temperature. Note that no nitrite formation was observed for deionized water solutions of DFO when deionized water solutions of FeCN were used. Nitrite formation requires the presence of PBS either in the DFO solution or in the FeCN solution. C) Nitrite formation

in 10 μM DFO solutions made in PBS, its individual composite reagents (NaCl, KCl, Na_2HPO_4 and KH_2PO_4), or deionized water after incubation with 80 mM FeCN for ten minutes at room temperature. D) Nitrite formation in 100 μM DFO (in deionized water) after incubation for up to 150 minutes at room temperature with 10 mM FeCN solutions made in commonly used laboratory buffer solutions. Nitrite formation in 100 μM DFO made in deionized water, or the primary composite reagents used to make various laboratory buffer solutions, without addition of FeCN (E), and following incubation of the different DFO solutions with 10 mM FeCN (F) for up to 60 mins at room temperature. Note that 10 μM of nitrite is formed in 60 minutes from 100 μM DFO solution in 10 mM CO_3^{2-} even without addition of 10 mM FeCN, and that no nitrite formation is observed in deionized water solutions of DFO even in the presence of FeCN. DFO solutions in PO_4^{2-} and PO_4^{3-} do not yield any nitrite unless FeCN is present.



E

Purge Vessel			NO
	I	II	
1.	H_2O	H_2O	Not detected
2.	PBS	H_2O	Not detected
3.	PBS	FeCN	Not detected
4.	FeCN	H_2O	**23 μM**

Figure 5.

NO rather than HNO is the reaction product of DFO and FeCN in PBS. A) Schematic diagram of the tandem purge vessel configuration. Two purge vessels were connected in series and continuously purged with argon gas (flow direction marked by red arrows). **B**) EPR measurement of the reaction product of DFO and FeCN. The first vessel was filled with 100 μM DFO and 100 mM FeCN, while the second one filled with 10 mM MGD and 0.5 mM Fe^{2+} in HEPES to capture the gaseous NO species produced in the first one. Incubation lasted for 15 min in dark under room temperature. The resulting solution in the second purge vessel was determined by EPR at room temperature. The triplet EPR signal of $(MGD)_2FeNO$ suggested the production of NO or HNO from the reaction of DFO and FeCN. C-E) Chemiluminescence measurement of the reaction product of DFO and FeCN. The second purge vessel was inline with a chemiluminescent NO analyzer. To test if

DFO generates HNO spontaneously or in reaction with FeCN, four protocols with different reagents in the two purge vessels were designed as shown in (E). C) ~50 μM of NO was recovered by chemiluminescence via protocol 3 following injection of 100 μM Angeli's salt which releases HNO, demonstrating the capability of this protocol to detect HNO. D) Representative voltage tracing of the four protocols following injection of 100 μM DFO into the first purge vessel. NO signal was measured only when DFO was in direct reaction with FeCN (protocol 4) but not when FeCN was downstream (protocol 3). These results suggest that DFO does not generate HNO, and that DFO directly reacts with FeCN.

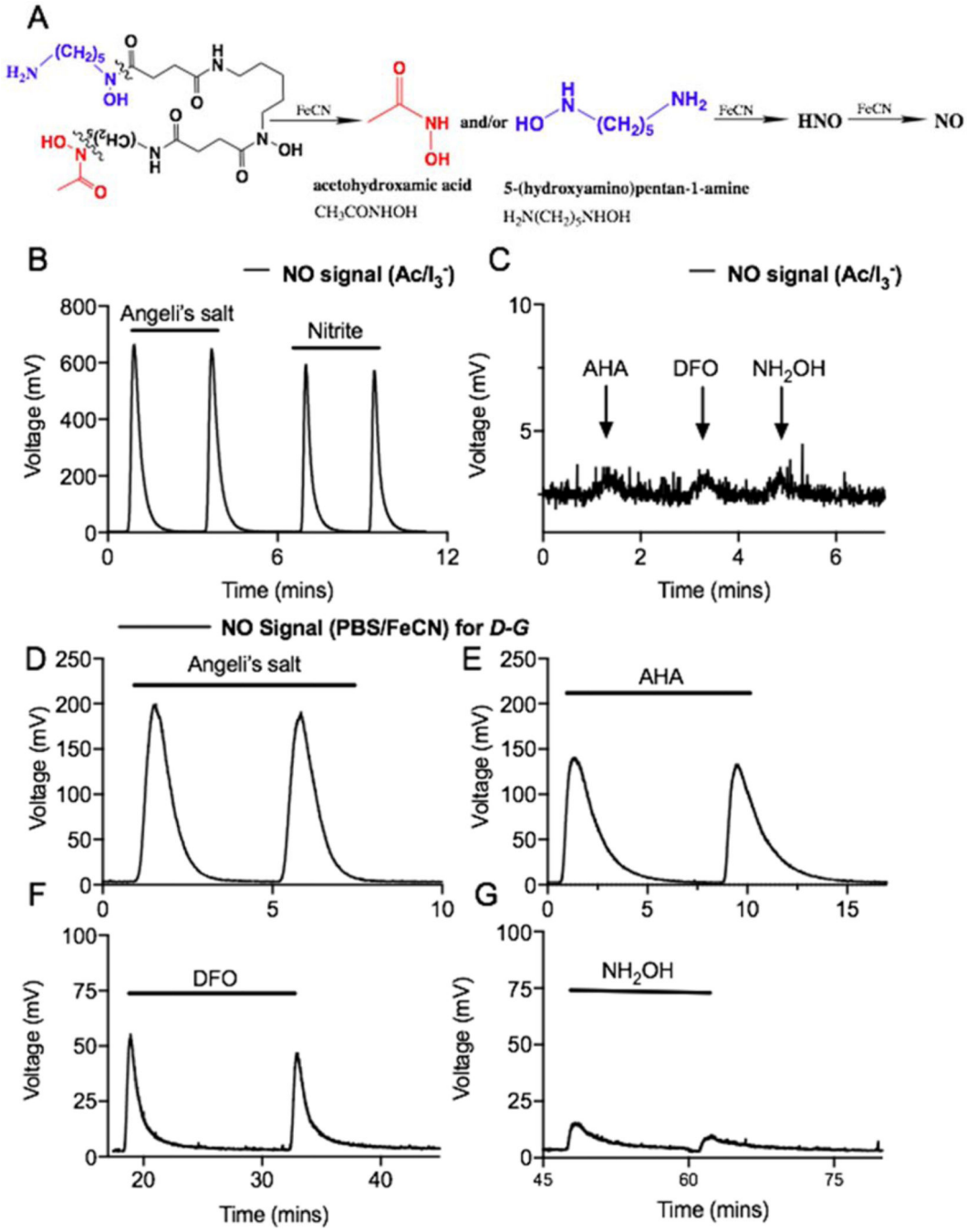


Figure 6.

Potential mechanism for the production of NO from DFO upon FeCN oxidation.

A) Schematic showing the possible intermediacy of acetohydroxamic acids or 5-(hydroxyamino)pentan-1-amine and nitroxyl in the oxidation of DFO to NO. NO generation from HNO (from Angeli's salt), acetohydroxamic acid and hydroxylamine was therefore characterized by chemiluminescence and compared to DFO. The generation of NO from Angeli's salt was compared to that of nitrite (B), and that of acetohydroxamic acid (AHA), deferoxamine (DFO) and hydroxylamine (NH_2OH) (C) in a reagent composed of acidified

triiodide (I_3^-). Nitrite and nitroxyl generate NO under I_3^- while AHA, DFO and NH_2OH do not. Nitrite does not generate NO under PBS/FeCN. On the other hand, nitroxyl (D), AHA (E), DFO (F) and NH_2OH (G) all generate NO under PBS/FeCN, albeit at different rates: Angeli's salt (HNO) > AHA > DFO > NH_2OH. The concentrations as well as rate of NO generation of each of the tested species are reported in Table 2. 10 μM of each NOx species was used, and all measurements were repeated in duplicates.

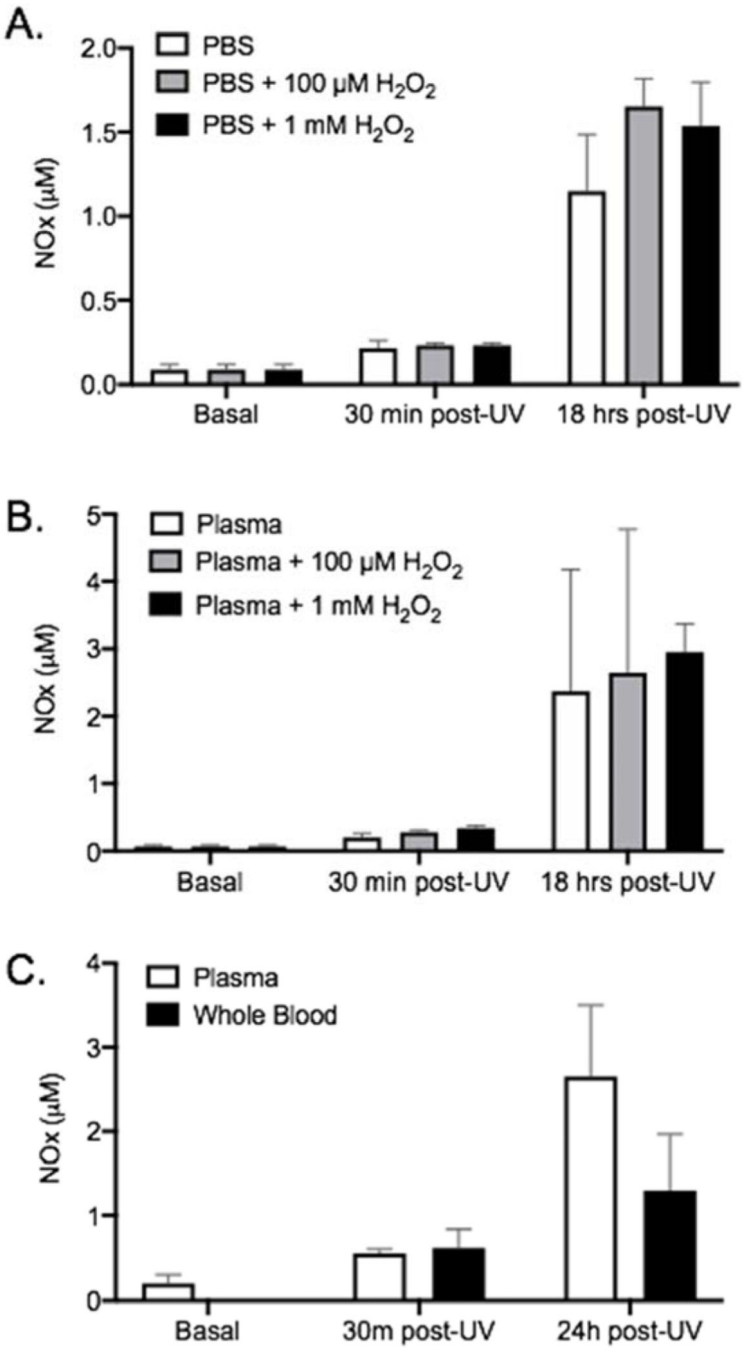


Figure 7.

NO production by DFO under UV light exposure. All measurements were performed in I_3^- reagent for total NOx determination. A) Nitrite formation from 100 μM DFO in PBS with and without H_2O_2 following 2h exposure to UV light. B) Nitrite formation from 100 μM DFO in sheep plasma with or without H_2O_2 following 2h exposure to UV light. C) NOx formation from 100 μM DFO in adult sheep whole blood vs. plasma following 2h exposure to UV light. The broad signal (not shown) from the whole blood suggested HbNO rather than nitrite formation in whole blood. Data is presented as mean ± SD (n = 3; number

of different animals). The less NOx measured in blood than plasma might result from the formation of undetectable nitrate from reaction of NO and oxyHb.

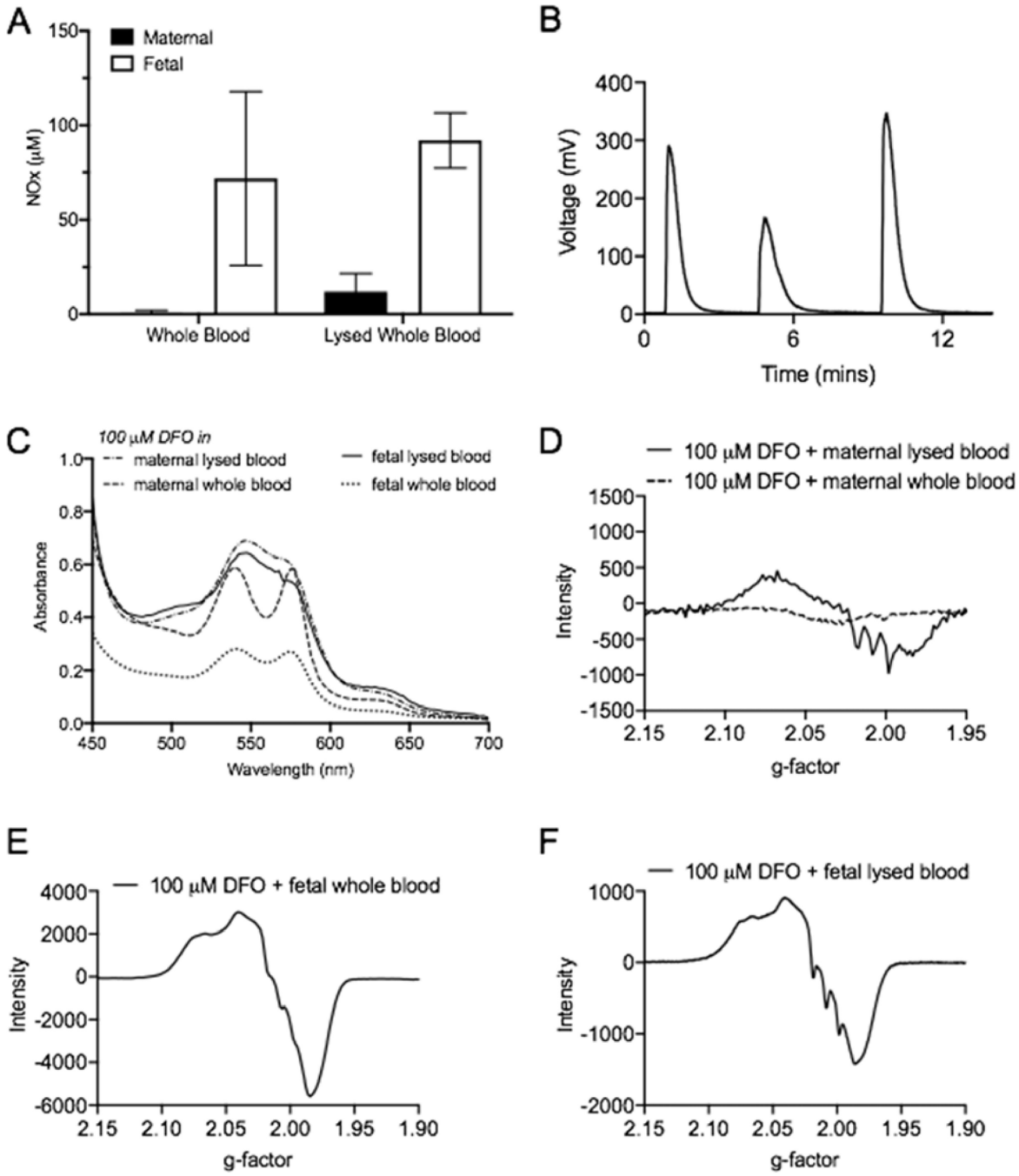


Figure 8.

NOx production from DFO in sheep adult and fetal blood. A) Quantification of the NOx concentrations, as determined by I_3^- chemiluminescence, produced when 100 μM DFO was incubated for 24 hours at 37 °C in adult and fetal sheep blood with or without lysis by repeated freeze-thaw. Data is presented as mean ± SD (n = 8; number of individual experiments from 3 sheep). B) Representative voltage tracing of the NOx signal obtained from samples of 100 μM DFO + fetal lysed blood injected into a purge vessel with I_3^-. The broad peaks were characteristic of HbNO formation, which was confirmed by UV-Vis

spectroscopy (C). HbNO formation was also confirmed with EPR for adult whole and lysed blood (D), fetal whole blood (E), and fetal lysed blood (F).

Table 1.

NO production by 10 μM HNO, acetohydroxamic acid, DFO and hydroxylamine under triiodide and FeCN conditions.

	AC/I_3^-			PBS/FeCN	
NOx	Conc. (μM)	Rate (pmol NO/min)		Conc. (μM)	Rate (pmol NO/min)
NO_2^-	9.9 ± 0.1	528 ± 22		N.D.	N/A
Angeli's salt (HNO + NO_2^-)	14.4 ± 0.2	454 ± 48		9.3 ± 0.5	223 ± 10
AHA	N.D.	N/A		13.7 ± 0.0	156 ± 18
DFO	N.D.	N/A		4.4 ± 0.1	37 ± 4
NH_2OH	N.D.	N/A		2.5 ± 0.2	16 ± 6

Data is presented as mean ± SD. N.D. = Not detected, N/A =Not available because signal was not detected.

Table 2.

NO production from DFO following 2 hours of UV exposure

	NOx (μM)		
***100 μM DFO in*:**	***Basal***	***30 mins post-UV***	***22 hrs post-UV***
PBS	0.09 ± 0.03	0.22 ± 0.05 †	1.2 ± 0.3 †
PBS + 100 μM H_2O_2	0.09 ± 0.03	0.23 ± 0.02 †	1.7 ± 0.2 ††
PBS + 1 mM H_2O_2	0.09 ± 0.03	0.23 ± 0.02 †	1.5 ± 0.3 †
Plasma	0.07 ± 0.02	0.20 ± 0.06 †	2.4 ± 1.8 †
Plasma + 100 μM H_2O_2	0.07 ± 0.02	0.28 ± 0.03 †	2.7 ± 2.1 †
Plasma + 1 mM H_2O_2	0.07 ± 0.02	0.34 ± 0.04 †	2.9 ± 0.4 †
Whole Blood	N.D.	0.6 ± 0.2 ††	1.3 ± 0.7 ††

Data is presented as mean ± SD.

† = $p < 0.05$ vs. basal

†† = $p < 0.01$ vs. basal