2025

Prevention of acute kidney injury by nitric oxide therapy in cardiac surgical patients: the role of nitrite and nitrate plasma levels

https://hdl.handle.net/2144/52241

BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

PREVENTION OF ACUTE KIDNEY INJURY BY NITRIC OXIDE THERAPY IN CARDIAC SURGICAL PATIENTS: THE ROLE OF NITRITE AND NITRATE PLASMA LEVELS

by

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B.A., Boston University, 2021

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science
2025

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ACKNOWLEDGMENTS

Thank you to the various research fellows who have come through the Anesthesia Center for Critical Care Research at Massachusetts General Hospital for their teachings and guidance throughout my years there. Binglan Yu, for her help with statistical analysis. Jamel Ortoleva, thank you for agreeing to participate in this project. Finally, Lorenzo Berra, for his mentorship and friendship over the past 7 years. Without you, I would not have stayed in medicine. Thank you.

PREVENTION OF ACUTE KIDNEY INJURY BY NITRIC OXIDE THERAPY IN CARDIAC SURGICAL PATIENTS: THE ROLE OF NITRITE AND NITRATE PLASMA LEVELS

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ABSTRACT

Background: Acute kidney injury (AKI) from cardiopulmonary bypass (CPB)-associated hemolysis is one of the most common complications from cardiac surgery. Considerable effort has been done by many to reduce AKI occurrence through the use of inhaled nitric oxide (iNO) therapy. iNO has been shown to reduce instances of AKI following CPB by limiting the NO scavenging activities of free hemoglobin. Nitric oxide (NO) is quickly metabolized into nitrite and nitrate once in plasma and considerable evidence exists that these metabolites may be exploited to increase NO's bioavailability further. This study aims to analyze the role plasma nitrite and nitrate concentrations play in reducing AKI in cardiac surgical patients treated with iNO during CPB.

Methods: 250 patients were enrolled in a clinical trial at Massachusetts General Hospital. Patients were divided evenly and received 80 ppm iNO nitric oxide or placebo gas during CPB and the 24 hours following the procedure. Of the 250 patients, 50 evenly distributed between treatment and control groups were identified and enrolled in this ancillary study to measure plasma nitrite and nitrate levels through chemiluminescence assay.

Results: Plasma nitrite and nitrate levels significantly increased in iNO-treated and placebo-treated patients 15 minutes, 24 hours, and 48 hours after CPB. No difference in AKI development or clinical biomarkers was found between the groups.

Conclusion: Plasma nitrite and nitrate levels successfully measure iNO administration. Additionally, evidence of an increase in nitrite and nitrate concentration following iNO therapy suggests its role in increasing the molecule’s bioavailability, potential mechanism of action for systemic NO delivery, and potential for therapeutic manipulation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

Ach	Acetylcholine
AKI	Acute Kidney Injury
BMI	Body Mass Index
cGMp	Cyclic Guanosine Monophosphate
CLD	Chemiluminescence Detector
CPB	Cardiopulmonary Bypass
CSICU	Cardiac Surgical Intensive Care Unit
ddH2O	Double Distilled Water
EDRF	Endothelium-Derived Relaxation Factor
eGFR	Estimated Granular Filtrate Rate
eNOS	Endothelial Nitric Oxide Synthase
Hb	Hemoglobin
HbFe(II)	Ferrous Hemoglobin
HbFe(III)	Ferric Hemoglobin
HbFe(III)-NO	Heme Nitrosyl
HCl	Hydrochloric Acid
HFNC	High-Flow Nasal Cannula
Hr	Hour
I^-	Sodium Iodide
I_2	Iodine
I_3^-	Triiodide

ICU	Intensive Care Unit
IFD	Intense Field Dielectric
iNO	Inhaled Nitric Oxide
KDIGO	Kidney Disease: Improving Global Outcome
LOS	Length of Stay
Met-Hb	Methemoglobin
MGH	Massachusetts General Hospital
mPAP	Mean Pulmonary Arterial Pressure
N_2	Dinitrogen Gas
$NaNO_2$	Sodium Nitrite
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric Oxide Synthase
OR	Operating room
Oxy-Hb	Oxygenated Hemoglobin
PAC	Pulmonary Artery Catheter
PAH	Pulmonary Arterial Hypertension
PPHN	Pulmonary Hypertension of the Newborn
PPM	Parts Per Million

PTFE	Polytetrafluoroethylene
RBC	Red Blood Cell
sGC	Soluble Guanylate Cyclase
VCl_3	Vanadium (III) Chloride
XOR	Xanthine Oxidoreductase

INTRODUCTION

Nitric Oxide: The Vascular Signaling Molecule

Originally termed endothelium-derived relaxation factor (EDRF), the correct identification of nitric oxide (NO) resulted in the 1998 Nobel Prize for Physiology or Medicine to be awarded to Robert Furchgott, Louis Ignarro, and Ferid Murad (Furchgott & Zawadzki, 1980; Ignarro et al., 1987). This endogenously generated molecule is responsible for vasorelaxation throughout the vasculature due to its activation of soluble guanylate cyclase (sGC). This activation results in increased intracellular cyclic guanosine monophosphate (cGMP), leading to the expulsion of calcium from smooth muscle cells and consequently causing vasodilation (Denninger & Marletta, 1999). The production of endogenous NO is achieved through the catalyzation of L-arginine via nitric oxide synthase (NOS) and the interaction of citrulline with oxygen and other cofactors (Balligand et al., 2009).

Endogenous Nitric Oxide Production: The Nitric Oxide Synthases

Three separate isoforms of NOS have been identified: neuronal NOS, inducible NOS, and endothelial NOS (eNOS), each responsible for the production of NO for specified activities such as neuronal regulation, immunological response, and vasculature regulation (Wood et al., 2013). Although each version of NOS has a uniquely vital role in NO regulation and overall homeostasis, this work will primarily focus on eNOS-derived NO and its effect on vasculature.

Under normal physiological conditions, eNOS is released from the endothelial cells that line the blood vessels in response to stimulation by acetylcholine (Ach). Once in the bloodstream, it catalyzes the reaction between L-arginine, oxygen, and a proton, resulting in the formation of NO (Lauer et al., 2001; Lundberg et al., 2008). Free NO is thought to have a half-life of approximately 0.05 to 1.8 milliseconds while in circulation, before undergoing 2eoxygenation. In the bloodstream, ferrous hemoglobin [HbFe(II)] binds with oxygen to form oxygenated hemoglobin (oxy-Hb). Oxy-Hb has a high affinity for NO and can bind to it during its short half-life, leading to the formation of ferric hemoglobin [HbFe(III)], also referred to as methemoglobin (met-Hb), along with nitrate (NO_3^-). Met-Hb cannot bind to oxygen and has a much lower affinity for NO compared to oxy-Hb (Doyle & Hoekstra, 1981; Kelm, 1999).

$$HbFe(II)\text{-}O_2 + NO \rightarrow HbFe(III) + NO_3^-$$

Illustration 1. Dioxygenation Reaction

The high affinity of oxy-Hb for NO results in it being considered an "NO scavenger," as it can effectively bind to NO, thus preventing it from interacting with the vasculature (Gladwin, Ognibene, et al., 2000). Unlike oxy-Hb's NO scavenging characteristics, met-Hb's low affinity for NO allows ferric proteins bound to NO to be further oxidized into nitrite (NO_2^-) through reductive nitrosylation. This process enables the oxidation of ferric heme nitrosyl [HbFe(III)-NO] to nitrite, which enhances the bioavailability of NO, as it becomes a more stable compound. This stability prevents oxy-

Hb from scavenging NO or losing its bioavailability due to the short half-life of NO (Cooper, 1999; Hoshino et al., 1996).

$$HbFe(III)\text{-}NO + OH^- \rightarrow HbFe(II) + HNO_2$$

Or

$$HbFe(III)\text{-}NO + H_2O \rightarrow HbFe(II) + HNO_2 + H^+$$

Illustration 2. Nitrosylation Reaction

Nitrite can increase the bioavailability of NO as nitrite can be further reduced to NO, particularly in periods of oxidative stress and hypoxemia via xanthine oxidoreductase (XOR), a nitrite reductase enzyme (Doyle et al., 1981; Lundberg et al., 2008; Millar et al., 1998). In the hypoxic state, less oxygen becomes available, thus inhibiting the ability of eNOS to interact with L-arginine to produce NO. To reduce the amount of ischemia and injury the body may experience, XOR can reduce nitrite into bioavailable NO until a normoxic state can be reinstated. Nitrite has also been shown to be produced in equal amounts to NO and is used as a direct measurement of bioavailable NO (Kelm, 1999; Lauer et al., 2001; Nagasaka et al., 2008).

Nitric Oxide Production: The Non-Canonical Pathway

Alternative pathways for NO production exist, such as the non-canonical pathway referring to NO production through the reduction of nitrate, into nitrite, and finally into bioavailable NO. As previously stated, endogenous NO goes through NO dioxygenation, producing large amounts of nitrate. Although chemically speaking, nitrate can be reduced into nitrite, the human body is unable to take part in such a reaction as it lacks the required enzyme to do so, leaving most of the plasma nitrate produced through NO metabolism to be excreted as waste (Lundberg et al., 2004, 2008). The majority of plasma nitrate comes directly from dietary supplements, such as lettuce, spinach, and other vegetables. Once ingested, oral bacteria immediately start absorbing nitrate, which is reduced to nitrite via nitrate reductase found in such bacteria. This results in a significant amount of nitrate, nitrite, and NO being present not only in the salivary glands but also in the stomach, with nitrate levels in the salivary glands being 10 to 20 times higher than those found in the bloodstream (Lundberg et al., 2004; Weitzberg et al., 2010).

Because of the non-canonical pathway, NO concentrations in the stomach are as high as 10-100 parts per million (ppm), significantly higher than in systemic vasculature. It is believed that such high levels of NO produced from enterosalivary circulation play a critical role in gastric health for immunological defense. Still, such details are beyond the scope of this work (Benjamin et al., 1994). Because nitrate reductase is present in the oral cavity and approximately 25% of all plasma nitrate comes from the oral cavity, it is safe to assume that large amounts of nitrate are reduced to nitrite and thus enter systemic circulation via oral absorption. Although the body has an inability to effectively take advantage of increased plasma nitrate levels, orally absorbed nitrite after nitrate reduction can act as an effective NO donor during periods of oxidative stress (Lundberg et al., 2008). Recent work has been done in order to exploit this pathway and increase NO availability during periods of severe hemolysis. The Nitrate-CIN trial, published within the past year, demonstrates that oral nitrate was effective in reducing instances of kidney injury by increasing plasma nitrite levels, thereby utilizing the non-canonical pathway for therapeutic purposes. Oral administration of inorganic nitrate was able to substantially raise the plasma nitrate (over 340 µM) and nitrite (over 1.40 µM) plasma concentrations. This drastic increase in NO metabolite concentrations sheds light on the amount of these compounds needed in the plasma to have the non-canonical therapeutic effect (Jones et al., 2024).

Nitric Oxide Bioavailability through Pharmacological Manipulation

In addition to nitrite reduction during periods of oxidative stress and/or hemolysis, the systemic vasodilatory effects of plasma nitrite have long been studied and well documented (Cosby et al., 2003; Gladwin, Shelhamer, et al., 2000; Weitzberg et al., 2010). Different vasodilators, such as nitroglycerine and nitroprusside, can exploit this pathway to produce NO once they reach their desired targets (Levy, 2005). This notion of using nitrite as a vasodilatory drug has also been shown through the use of nebulized sodium nitrite for effectively treating pulmonary arterial hypertension (PAH); however, treatment with this drug exhibited symptomatic hypotension (Hunter et al., 2004; Rix et al., 2015). In addition to the systemic drops in blood pressure observed with these medications, exactly how much nitrite is being converted to NO has not been tested and remains unclear.

In contrast to nitrate/nitrite derived drugs, inhaled nitric oxide (iNO) has been shown to be a selective pulmonary vasodilator (Morris & Rich, 1997). Originally believed to be toxic for the body, Warren Zapol and his team were able to treat infants with persistent pulmonary hypertension of the newborn (PPHN) by administering 80 ppm of iNO to these “blue babies.” The infants were successfully treated with almost immediate results. They showed a significant oxygen saturation increase with no changes in systemic blood pressure or met-Hb levels (Roberts et al., 1992). This pioneering demonstration resulted in iNO receiving FDA approval. It quickly started a race towards discovering the different therapeutic tools iNO had to offer, resulting in significant research being conducted to expand its use. To date, iNO is experimentally used as part of the treatment plan for adult and pediatric patients going through/experiencing acute respiratory distress syndrome, myocardial infarction, cardiac surgery, and more recently its role as an antimicrobial agent. Unlike nitrate/nitrite derived drugs, iNO is short acting due to NO’s short half-life, does not require enzymatic changes, and does not affect systemic blood pressure unlike those previously described in this section (Di Fenza et al., 2023; Lei et al., 2018; Liu et al., 2007; Nagasaka et al., 2008; Redaelli et al., 2023; Wiegand et al., 2021).

Endothelial Function and its Role in Nitric Oxide Regulation

Proper endothelial function refers to the vasculature endothelium releasing eNOS when stimulated by ACh. This leads to the catalytic reaction between L-arginine and oxygen to create NO, which can then initiates vasodilatation of the surrounding vasculature (Deanfield et al., 2007). For instances where the endothelial cell is physically or physiologically damaged, loses integrity, becomes inert, or is entirely absent, the tunica media of the vessel will be unable to relax due to the absence of NO, resulting in hypertension of the area. Whether the issue involves the damage or inappropriate activation of the endothelial cell in the tunica intima, missing component in the eNOS-L-arginine pathway, or lack of bioavailable substrates needed for NO production, the absence of NO production can result in significant trauma and contribute to cardiovascular risk factors. Additionally, enhanced cardiovascular risk factors such as smoking and a poor diet can cause overactivation and recruitment of the endothelial cells, leading to a decrease in physical and physiological integrity, resulting in the development of other cardiovascular risk factors and a reduction in NO bioavailability (Deanfield et al., 2007; Kleinbongard et al., 2006). Although many attributes could be to blame for the inappropriate functioning of the endothelial cell, no primary culprit has been identified and is likely variable from person-to-person. Given this, the term "endothelial dysfunction" will be used to characterize the inappropriate function of the endothelial cell.

Considerable evidence exists illustrating the eNOS and NO are essential for proper vasoactive responses. Work by Woods and colleagues shows that eNOS-deficient mice have higher blood pressures than eNOS-sufficient mice. Still, eNOS-deficient mice experienced a decrease in healthy blood pressure levels when bone marrow from eNOS-positive mice was transplanted into eNOS-deficient mice (Wood et al., 2013). Work by Lauer has shown how ACh stimulated eNOS in healthy patients actually increased plasma nitrite concentrations, highlighting NO release from the endothelial cell (Lauer et al., 2001). Finally, work by Kleinbongard has shown how plasma nitrite levels not only decrease in the presence of a dysfunctional endothelium, but also mirrors eNOS activity and consequently decrease in concentration alongside the cardiovascular risk factors increase (Kleinbongard et al., 2006).

The Nitric Oxide and Erythrocyte Relationship

As previously stated, plasma nitrite functions to increase bioavailability levels. Although nitrite is chemically more stable than NO, it is still considered an unstable molecule with a short half-life of only a few minutes. It is believed that only 1/3 of the total nitrite concentration is actually in the plasma, and the remaining 2/3 is found within the red blood cell (RBC) (Kleinbongard et al., 2006). Evidence suggests that RBCs contain eNOS and can produce both NO and nitrite in hypoxic environments. The RBC's ability to create and release NO in a moment of oxidative stress suggests the cell plays a role in NO metabolism and can increase NO bioavailability in periods of oxidative stress (Cosby et al., 2003; Crawford et al., 2006; Webb et al., 2008). It is important to mention that intravascular hemolysis produces similar effects to those of baseline endothelial dysfunction. Free-floating hemoglobin's high affinity for NO both in the free and oxygenated state makes it an NO scavenger, reducing plasma NO and thus resulting in the same physiological effects noted in patients with endothelial dysfunction. iNO has been used as an excellent tool to alleviate free Hb scavenging qualities seen during periods of severe hemolysis (Minneci et al., 2005).

Hemolysis in the Vasculature

Hemolysis-induced endothelial dysfunction, for lack of a better term or description, will have physiological effects similar to normal endothelial dysfunction. During hemolysis, the erythrocyte is either fully or partially damaged, leading to the changes in cell structure or complete apoptosis. As this occurs, hemoglobin is released and is termed free hemoglobin. Once free oxy-Hb is floating in the plasma, it takes part in the normal dioxygenation reaction and thus depletes plasma NO levels (Schaer et al., 2024; Spina et al., 2019). In normal physiological conditions, that is those with low hemolytic activity, haptoglobin and hemopexin scavenge free hemoglobin and heme protein released during cell lysis and are destroyed via phagocytosis by splenic and liver macrophages (Schaer et al., 2024; Spina et al., 2019). During excessive or prolonged hemolysis, heme scavenging cells become overwhelmed, resulting in excess free hemoglobin depleting plasma NO. The system is then forced to rely on renal filtration to excrete unwanted byproducts, leading to renal hypertension and tubular injury (Spina et al., 2019). This is not a novel discovery and has been studied extensively by the Gladwin group through a sickle cell model (Kato et al., 2017).

Hemolytic activity has been well documented through studies of erythrocytes during blood transfusion (Edgren et al., 2010; Weinberg et al., 2008). This is likely a result of the physical damage the cells undergo during donation or transfusion, similar to how cardiopulmonary bypass machine-associated hemolysis occurs due to the velocity of the cardiopulmonary bypass machine (Berra et al., 2012; Schaer et al., 2024). The Berra group tested this by administering autologous, leukoreduced packed RBCs stored for 3 or

40 days in healthy volunteers. They were able to show increased rates of hemolysis in patients who received RBCs stored for 40 days and in plasma hemoglobin, meaning there is an increase in NO scavenging activity. Interestingly, no changes in blood pressure were noted, and an increase in plasma nitrite was also observed. Berra theorized that due to these patients having a healthy endothelium, there might have been an increase in eNOS activity in order to overcompensate for the increase in free hemoglobin scavenging NO (Berra et al., 2012). To further test this, the Berra group recreated the study using patients with confirmed endothelial dysfunction, with the additive factor of administering iNO (Berra et al., 2014).

As previously noted, iNO directly increases bioavailable NO and does not affect systemic blood pressure, unlike other NO producing drugs. Unlike in the healthy patient study, the group found that patients with endothelial dysfunction had an increase in blood pressure after 40-day stored erythrocyte administration. They noted no increase in plasma nitrite levels, making it safe to assume that the endothelium was unable to produce NO or keep up with the increase in free hemoglobin. In contrast, the study group administered RBCs stored for the same duration and treated with 80 ppm of iNO showed no increase in blood pressure, an increase in plasma NO metabolites, and a reduction in NO consumption from free hemoglobin. It was noted that this therapy did not yield dangerous levels of met-Hb accumulation in the vasculature and demonstrated that iNO therapy was effective in reducing the harmful effects of hemolysis in patients with endothelial dysfunction via supplemental NO administration (Berra et al., 2014).

Cardiopulmonary Bypass-Associated Hemolysis

High rates of hemolysis are also noted among patients undergoing procedures involving cardiopulmonary bypass (CPB). CPB is used during cardiothoracic surgery when the surgeon must arrest the heart and is commonly referred to as a “heart-lung machine.” CPB is often used during valve replacements/repairs, heart and lung transplants, and aortic repairs (Spina et al., 2019). During CPB, the aorta is cross-clamped, and blood is cycled through the CPB machine, where it becomes oxygenated. Although large pumps within the CPB circuit push the blood to create a flow rate compatible with life, the physical pumps are not believed to be a contributing factor for cell hemolysis. Instead, the shear stress from the velocity of the blood flowing through the circuit damages the cells and results in the hemolysis observed during these procedures (Schaer et al., 2024). Because of this, patients with suspected endothelial dysfunction are those most at risk for any cardiac surgical procedure, specifically those involving CPB. These risk factors include, but are not limited to: diabetes mellitus, hypertension, obesity, atherosclerosis, peripheral vascular disease, hyperlipidemia, and smoking (Marrazzo et al., 2019).

CPB-Associated Hemolysis: Acute Kidney Injury

As previously stated, hemolysis due to CPB releases oxy-Hb, increasing the rate of NO consumption by hemoglobin, reducing bioavailable NO, and increasing plasma met-Hb which is nephrotoxic. Because of the amount of hemolysis that occurs as a result of CPB, acute kidney injury (AKI) has been found to be the most common and severe postoperative complication related to cardiac surgery (Spina et al., 2019). Identification of any renal injury is often delayed compared to what happens on a real-time physiological level due to our reliance on laboratory biomarkers. AKI is noted by an increase in serum creatinine, a decrease in urine output, or both. Multiple identifications for AKI exist, with the Kidney Disease: Improving Global Outcomes (KDIGO) criteria being the most widely used as it focuses on more minor changes in these biomarkers. Anywhere from 30-70% of patients undergoing CPB will develop AKI and approximately 20% of these patients will require renal replacement therapy. Post-CPB AKI is not just a result of CPB-associated hemolysis, but also a result of fluid resuscitation in the postoperative setting, aortic cross-clamp time, use of different blood products, interaction of multiple medications, fat and/or blood embolism, inflammation, or sepsis (Schaer et al., 2024; Spina et al., 2019). Because of the severity and occurrence of AKI following cardiac surgical procedures, it is of great clinical importance to find a way to reduce AKI during procedures requiring CPB.

Treatments for CPB-Associated Hemolysis: The Nitric Oxide Studies

Multiple clinical trials aimed at improving the safety and efficacy of cardiac surgery have used iNO as a successful intervention during CPB. iNO has been found to have significant cardioprotective qualities during these procedures (Gianetti et al., 2004; Kamenshchikov et al., 2019). In a clinical trial published in 2019, patients undergoing CPB treated with 40 ppm iNO were found to have lower myocardial injury markers than those who were not. Additionally, patients treated with iNO did not require the same degree of postoperative fluid resuscitation as those not treated with iNO (Kamenshchikov et al., 2019). This same methodology has been used to reduce AKI rates in cardiac surgery. Kamenshchikov and colleagues developed a randomized controlled clinical trial where iNO or placebo was administered during CPB to 96 patients with the aim of reducing AKI rates post procedure. They found AKI to be significantly decreased in the study group compared to the control group (20.8% vs. 41.6%; $p = 0.23$) (Kamenshchikov et al., 2022). It is important to note that with this study, iNO was only administered during CPB and enrolled patients were not screened for endothelial dysfunction. Similar in methodology, Lei and colleagues performed a similar clinical trial aimed at reducing post-CPB-AKI and improving renal outcomes in healthy Chinese patients. For this study, they enrolled 244 otherwise healthy individuals undergoing multi-valve replacement surgery, primarily as a result of rheumatic fever. Unlike the Kamenshchikov studies, the Lei study administered 80 ppm iNO from the beginning of CPB and concluded 24 hours post CPB. Again, results were significant and found the NO group had a substantial difference in AKI rates as compared to the placebo group (50% vs. 64%; $p = 0.014$), reduction in stage 3 chronic kidney (21% vs. 33%; $p = 0.024$), and better renal outcome 1 year post CPB procedure ($p = 0.041$). An important note from this study, which should not be overlooked, is that prolonged iNO administration at 80 ppm was shown to be safe. Both met-Hb and NO_2, toxic byproducts of NO, always remained within safe levels, and at no time was iNO decreased or stopped for fear of patient safety (Lei et al., 2018). Although this groundbreaking study illustrated the therapeutic effect of iNO in reducing AKI during CPB and showed that the delivery of prolonged iNO was indeed safe in humans, a large limitation of this study is the young and healthy population that was used. Even though 244 patients were enrolled, these were patients with little to no comorbidities, which is not adequately translated to the western population. Often, people electing to participate in non-emergent cardiac procedures have some cardiac risk factors, mainly attributed to endothelial dysfunction (Lei et al., 2018; Marrazzo et al., 2019).

Using inspiration and knowledge learned from the Lei et al, a single-center, double-blinded, placebo-controlled clinical trial was initiated at Massachusetts General Hospital (MGH). This trial aimed to reduce AKI in cardiac surgical patients, with endothelial dysfunction, through iNO therapy during and continuing after CPB. Patients with endothelial dysfunction present increased risk for severe outcome during cardiac surgery (Marrazzo et al., 2019). Based on prior work listed throughout, it is hypothesized that treating this patient population with iNO during the cardiac surgical procedure will provide significant therapeutic effect and reduce instances of severe renal outcome. This work aims to study the role plasma nitrite and nitrate play in the therapeutic effect of iNO in cardiac surgical patients with confirmed endothelial dysfunction. Given both compounds are primary byproducts of NO metabolism and also serve to increase the bioavailability of NO, it is believed that an increase in plasma nitrite and nitrate levels should enhance the therapeutic effect of iNO, thus reducing instances of renal injury and improving long term outcome. Additionally, successful identification of increased nitrite and nitrate levels will serve as confirmation of successful NO increase through iNO delivery.

METHODS

The current work is an ancillary study, a derivative of a clinical trial by Berra and colleagues. This clinical trial was approved by the Institutional Review Board of the MGH (IRB No: 2016P001629) and registered on *Clinicalrials.gov* (NTC02836899). The results from the clinical trial have not yet been published and this ancillary work will not focus on the trial's primary endpoint.

Clinical Trial

This single-center, double-blinded, placebo-controlled clinical trial was approved by our institution's IRB and monitored by the Data Safety Monitoring Board. The primary endpoint was to reduce AKI in patients undergoing CPB through NO therapy. Marrazzo et al. thoroughly explains the clinical trial procedure, which is summarized below (Marrazzo et al., 2019).

Enrollment consisted of 250 patients evenly randomized between the control (nitrogen gas) and treatment (NO) groups. All inpatients and outpatients scheduled to undergo cardiac surgery were screened. Patients meeting inclusionary criteria were approached during a pre-operative appointment after permission was obtained from the Attending Surgeon. Males and females over the ages of 40 and 50, respectively, who were undergoing elective cardiac or aortic surgery requiring the use of CPB for a period of 90 minutes or longer, had stable renal function and exhibited signs of endothelial dysfunction, were eligible to participate in this clinical trial. Patients with an estimated granular filtrate rate (eGFR) greater than 30mL/min/1.73m^2, mean pulmonary arterial pressure (mPAP) greater than or equal to 40 mmHg, and/or had received intravenous contrast infusion(s) within 48 hours of their scheduled procedure were excluded from participation. Eligible patients were asked to complete a questionnaire to gauge their level of endothelial dysfunction, if patients were found to not have endothelial dysfunction, they were automatically excluded from the study. Patient recruitment for this clinical trial took place starting in February of 2017 and was supposed to conclude in May of 2020. During the early months of 2020, MGH postponed all elective surgeries, significantly affecting the primary study. Patient enrollment resumed once it was deemed safe, and data collection was finally concluded in October of 2024. Please see Marrazzo et al. for a more detailed look at inclusion and exclusionary criteria (Marrazzo et al., 2019).

Written and informed consent was gathered by a study team member who would not be part of the patient's medical care. All members of the clinical and research teams were blinded as to which treatment group the patient would be randomized into, until a 1-year follow-up occurred. Due to safety concerns, the member of the research team responsible for gas delivery would not be blinded, and the principal investigator could unblind the clinical team when the responding clinician responsible for the patient deemed it necessary.

Once in the operating room (OR) and the patient was placed under general anesthesia, a pulmonary artery catheter (PAC) was placed by the medical team and final eligibility was confirmed based on the mPAP. If patients were found to have a mean pulmonary artery pressure equal to or greater than 40 mmHg, the patient was automatically excluded and would not be randomized. Exclusion from the clinical trial had no merit in medical decisions made by the medical team. Those patients still eligible to participate would be randomized into either study group, until an equal number of participants was present in both groups (125 control, 125 treatment).

Study gas delivery through CPB was initiated by the unblinded study team member once CPB commenced and would continue for a period of 24 hours (hrs), through mechanical ventilation. In the event that the patient were to be extubated before the allocated study time, study gas would continue to be delivered via high flow nasal canula (HFNC). While the patient was under CPB, and for the 24 hours after CPB was discontinued, the patient was administered 80 ppm of iNO or dinitrogen gas (N_2). While being oxygenated through CPB, 80 ppm of iNO delivery was achieved through the following equation:

$$\frac{\left[sweep\ flow\ \left(\frac{L}{\min}\right) x\ NO\ concentration\ (80\ ppm)\right]}{NO\ tank\ concentration\ (850\ ppm)}$$

Illustration 3. CPB-NO Administration Equation

Further details regarding gas delivery through the CPB oxygenator and once in the cardiac surgical intensive care unit (CSICU) can be found in Marrazzo et al. (Marrazzo et al., 2019). At the 24 hour mark, patients were gradually weaned from iNO/N_2 rather than abruptly taken off the gas, given the high risk of rebound pulmonary hypertension, and other severe consequences that can be experienced when acutely taken off iNO (Atz et al., 1996; Black et al., 1999; Schulze-Neick et al., 1999).

Blood and urine samples were collected from the patient at five timepoints: (A) baseline sample before CPB, (B) 15 minutes after cessation of CPB, (C) 24 hrs after CPB, (D) 48 hrs after CPB, (E) 6 weeks after surgical procedure. Once blood samples were collected, plasma was separated from the RBCs via centrifuging, snap frozen using liquid nitrogen, and stored in specified locations in freezers at negative 80°C. Samples were stored until ready for analysis, as deemed safe to do so based on evidence from prior works (Basu et al., 2022). For further details regarding the storage and handling of urine, please see selected works (Marrazzo et al., 2019).

Nitric Oxide Measurements: The Chemiluminescence Approach

Multiple methods for measuring NO exist, mainly through measurements of NO metabolites. The Griess assay has historically been relied upon for NO measurements due to its sensitivity for low concentrations (Basu et al., 2022). This method relies on the compound's reaction with sulfanilamide and N-(1-naphthyl) ethylenediamine under acidic conditions (Kleinbongard et al., 2002). Although the Griess assay is relatively simple and inexpensive to use, this process has difficulty measuring samples with low concentrations and directly measures nitrite. Because NO and nitrite have a direct correlation in plasma level, nitrite measurements can be accounted for by the NO concentration measured through the Griess assay. Additionally, the assay relies on the fact that nitrite is being reduced to NO under acidic conditions, not nitrate (Basu et al., 2022; Kelm, 1999; Lauer et al., 2001; Weitzberg et al., 2010). Nitrate can still be measured under the Griess assay but only after the addition of a nitrate reductase to the reaction. The final nitrate concentration will then be obtained by subtracting the new nitrite concentration from the original nitrite concentration (Basu et al., 2022). Given the Griess assay's preference for low nitrite concentration samples and extra steps required for nitrate measurements, it does not serve as a reliable format for plasma nitrite and nitrate sample analysis.

The most precise form of NO measurement comes from the chemiluminescence-based assay (Basu et al., 2022; Di Fenza et al., 2022). This form of NO measurement also relies on the reduction of different NO metabolites (nitrite, nitrate, S-nitrosohemoglobin, and iron-nitrosyl complexes) found in liquid or tissue samples. This is accomplished by the sample's interaction with ozone to turn the sample (i.e. nitrite), into an excited state, causing photon emission directly measurable to the sample's NO concentration, which is then measured by the chemiluminescence detector. Specifically with liquid samples, the detector is directly connected to a system containing the appropriate reaction needed to measure the specified sample (nitrite, nitrate, etc.), and is continuously purged with an inert gas, typically nitrogen. Figure 1 illustrates the glassware needed to accomplish this process (Di Fenza et al., 2022).

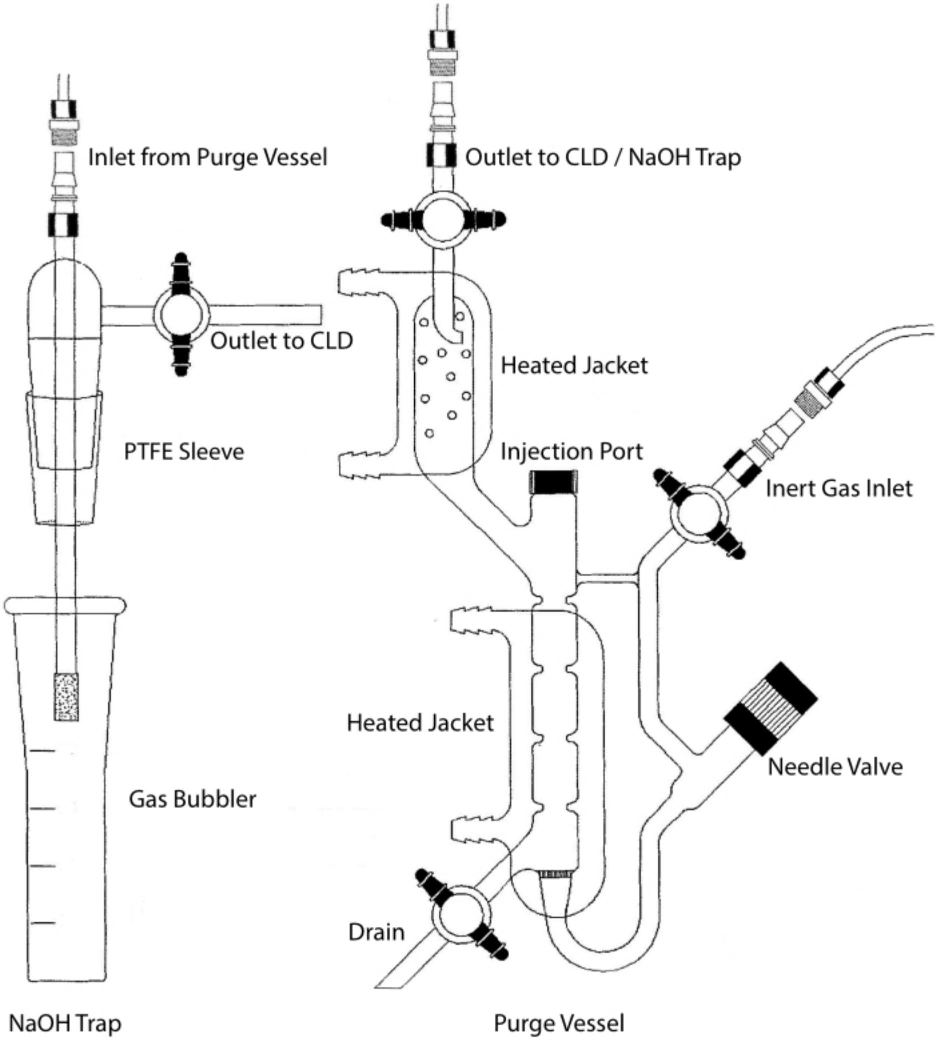

Figure 1. Required Glassware for Nitric Oxide Detection through Chemiluminescence Assay Detection of NO will occur through the purge vessel, illustrated on the right, as injected samples, injected through the injection port, interact with sample-dependent solution within the purge vessel. The heated jacket allows for hot water to flow through the purge vessel's surrounding, necessary for detecting nitrate samples via vanadium (III) chloride (VCl_3) in hydrochloric acid (HCl) assay. The needle valve allows for chamber pressure control. The inert gas inlet allows for inert gas, usually nitrogen, to be delivered from a connected source to purge the vessel at 3 -5 ppm. Because of the corrosive nature of HCl, a sodium hydroxide (NaOH) trap is necessary during VCl_3 in HCl assay. Intense field dielectric (IFD) filter is used between the glassware chemiluminescence detector (CLD). PTFE = polytetrafluoroethylene. *Source: Adapted from Di Fenza et al., 2022.*

Chemiluminescence-based Nitrite Detection

Detection of nitrite through chemiluminescence-based assay has proven to be the most reliable and sensitive method for NO detection through nitrite reduction, with the specific procedure being used in this study being adapted from Di Fenza et al. (Basu et al., 2022; Di Fenza et al., 2022). This is accomplished by creating a low pH environment, similar to that exercised during hypoxic conditions, resulting in nitrite reduction into NO. Measurements were done through 20µL injections of plasma through the injection port of the purge vessel, into a triiodide (I_3^-) solution. The specified equation, as noted by Di Fenza et al., is shown below:

$$I_3^- \rightarrow I_2 + I^-$$

$$2NO_2^- + 2I^- + 4H^+ \rightarrow 2NO + I_2 + 2H_2O$$

$$I_3^- + 2RS\text{-}NO \rightarrow 3I^- + RSSR + 2NO^+$$

$$2NO^+ + 2I^- \rightarrow 2NO + I_2$$

Illustration 4. Nitrite-Triiodide Chemiluminescence Reaction

Precautions were taken when preparing the I_3^- solution due to the use of corrosive compounds, and work was conducted under a fume hood. Once prepared, the solution was stored at 4°C and for a period not exceeding 7 days. Preparation of the I_3^- solution was accomplished by thoroughly mixing the following compounds for a minimum of 30 minutes:

1. Sodium iodide (I^-): 2.0 g.

2. Double distilled water (ddH_2O): 40 mL.
3. Iodine (I_2): 1.3 g.
4. Acetic acid: 140 mL.

In order to correctly measure nitrite samples, a nitrite stock solution was prepared to serve as a baseline for nitrite measurements. The nitrite stock solution was produced in the following manner:

1. Production of 100 mM sodium nitrite ($NaNO_2$) through dissolution of 0.69 g sodium nitrite ($NaNO_2$) and 100 mL ddH_2O.
2. 100 mM $NaNO_2$ dilution into 1mM $NaNO_2$ by taking 1mL of 100 mM $NaNO_2$ and diluting with 100 mL ddH_2O.
3. 1 mM $NaNO_2$ dilution into 5 µM $NaNO_2$ by taking 0.5 mL of 1mM $NaNO_2$ and diluting with 100 mL ddH_2O.
4. 5 µM $NaNO_2$ dilution into 2 µM $NaNO_2$ by taking 10 mL of 5 µM $NaNO_2$ and diluting with 25 mL ddH_2O.
5. 2 µM $NaNO_2$ dilution into 1 µM $NaNO_2$ by taking 12.5 mL of 2 µM $NaNO_2$ and diluting with 25 mL ddH_2O.
6. 1 µM $NaNO_2$ dilution into 0.50 µM $NaNO_2$ by taking 12.5 mL of 1 µM $NaNO_2$ and diluting with 25 mL ddH_2O.
7. 0.50 µM $NaNO_2$ dilution into 0.25 µM $NaNO_2$ by taking 12.5 mL of 0.5 µM $NaNO_2$ and diluting with 25 mL ddH_2O.

Once a stock solution was prepared and the assay was running, a standard curve was created using baseline $NaNO_2$ samples of 0.25 µM, 0.50 µM, 1 µM, 2 µM, 5 µM samples. All injections for nitrite measurement were 20 μL, due to the low concentration of nitrite found in plasma compared to other compounds. Each nitrite sample was injected two separate times to account for proper procedure. Once the plasma sample is injected into the vessel, it reacts with the acidic solution, generating NO, which will be detected by the chemiluminescence detector (CLD). The Sievers 280i Nitric Oxide Analyzer served as the CLD for this study.

The reaction between the I_3^- solution and plasma nitrite is unstable and produces significant foaming when plasma samples are injected, thus it must be changed if the reaction space is surpassed by the solution. Changes in the environment within the purge vessel, including but not limited to the adjustment of inert gas delivery, purge vessel pressure, development of sedimentary impurities, could result in inaccurate readings. Any time a change within the purge vessel occurred, the experiment was stopped, the vessel was thoroughly cleaned using ddH_2O and sodium hydroxide (NaOH), new I_3^- solution added, and a new calibration curve once again acquired. A foaming solution was added to limit/prevent such events. Each sample was injected and analyzed against the calibration curve used for such specific solution and sample. Once the CLD detects a sample, it will produce a peak observed via the CLD computer program.

During off-line analysis, the values of each injection were calculated based on the area under the curve produced by each injection. The values for the baseline $NaNO_2$ stock solution serve as the calibration solution, which creates a curve with a linear $y = mx + q$ equation. The concentration of the two plasma nitrite sample injection values were averaged and plotted onto the following equation for proper measurement:

$$NO\ Consumption\ (\mu M) = \frac{Area - q}{m}$$

Illustration 5. Off-Line Analysis Equation

Chemiluminescence-based Nitrate Detection

Similar in background and methodology, nitrate levels were measured using the chemiluminescence assay approach using the procedure outlined by Di Fenza et al. (Di Fenza et al., 2022). I_3^- solution is able to be used for nitrate measurements, but only if nitrate reductase is added, and creates similar issues as noted with the Griess assay (Basu et al., 2022). Vanadium (III) chloride (VCl_3) in hydrochloric acid (HCl) was used as the solution required for the reaction. For the reaction to proceed properly, the purge chamber needed to be at a continuous temperature of 90°C, achieved through the heated jacket of the purge vessel. Measurements were done through 10 µL injections from the plasma samples, rather than 20 µL, due to the large concentrations of nitrate present in plasma. The specific equation is shown below, as noted by Di Fenza et al.:

$$2NO_3^- + 3V^{3+} + 2H_2O \rightarrow 2NO + 3VO_2^+ + 4H^+$$

Illustration 6. Nitrate-Vanadium (III) Chloride Chemiluminescence Reaction

Significant care was used when preparing the solution due to the photosensitivity nature of the reaction and toxicity of the compounds involved. Additionally, the exothermic properties of this reaction required the solution to be produced in a specific order. Once prepared, the solution was be stored at 4°C and for a period not exceeding 2 weeks. Preparation of the VCl_3 solution is as follows:

1. 1.6 g (or 0.8 g) of VCl_3 is added to a clear container wrapped in aluminum foil.
2. Addition of 200 mL (or 100 mL) 1 N HCl.
3. Mix for 30 minutes.
4. Filter through Whatman #1 vacuum filter.
    a. Due to the photosensitive nature of the reaction, both the upper portion and collection portion of the Whatman filter was covered in aluminum foil.

As with the nitrite chemiluminescence procedure, nitrate samples required a stock nitrate solution made from sodium nitrate ($NaNO_3$) to produce an adequate baseline sample calibration curve. The following was done to achieve this:

1. Preparation of 100 mM sodium nitrate ($NaNO_3$) through dissolution of 0.85 g $NaNO_3$ and 100 mL ddH_2O.
2. 100 mM dilution into 1mM $NaNO_3$ by taking 1mL of 100 mM $NaNO_3$ and diluting with 100 mL ddH_2O.
3. 1mM $NaNO_3$ dilution into 200 µM $NaNO_3$ by taking 5 mL of 1mM $NaNO_3$ and diluting with 25 mL ddH_2O.
4. 200 µM $NaNO_3$ dilution into 100 µM $NaNO_3$ by taking 12.5 mL of 2 µM $NaNO_3$ and diluting with 25 mL ddH_2O.
5. Repeating this process for 50 µM, 25 µM, and 12.5 µM concentrations.

The purge vessel was prepared in the same fashion as it was for the nitrite samples, with the additive factor of needing to maintain the purge vessel at a temperature of 90°C throughout the duration of the experiment.

Once the VCl_3 solution was prepared, it was added to the purge vessel prior to heating of the vessel. This was done because of the possibility of a violent reaction occurring from VCl_3 at 4°C and the 90°C vessel. Once the reaction was at the proper temperature, the blue reaction would change color to light green, indicating a successful reaction. Just as with the nitrite samples, a calibration curve will be made each time there is a change to the solution or if the solution surpasses the reactionary space within the vessel. Even though this solution is much more stable than the I_3^- solution, a foaming solution was still added. Once the system was functioning properly, a calibration curve using the following $NaNO_3$ concentration was created: 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM.

Each plasma nitrate sample was injected twice to account for proper procedure. Of note, treatment group samples collected at the 24 hr and 48 hr time points were diluted using 50 µL of plasma and 150 µL of ddH_2O into a separate microcentrifuge tube before chemiluminescence assay. In one instance, one sample collected at the 15-minute post-CPB timepoint, had to be diluted in the same format. This was done because of the high likelihood these specific samples had significantly high nitrate concentrations. Please see the concurrent subsection regarding sample acquisition for further details.

Nitrate samples were analyzed in the same fashion as the nitrite sample during off-line analysis. Please see preceding subsection for details regarding off-line analysis.

Sample Collection and Identification

Blood samples were collected via EDTA-coated tubes in 250 patients enrolled in the clinical trial in the following timepoints: (A) baseline after PAC placement, (B) 15 minutes after cessation of CPB, (C) 24 hrs after CPB, (D) 48 hrs after CPB, (E) 6 weeks after CPB. For this particular study, it was decided to measure only time points A, B, C, and D, due to the half-life of these compounds and the ultimate goal of identifying the role that plasma nitrite and nitrate samples play in reducing AKI in this patient population, which occurs within 48 hours of CPB cessation.

Once blood was collected in the OR or CSICU, one member of the study team had to immediately return to the lab and centrifuge the sample, given the short half-life of these compounds. All samples underwent centrifugation and plasma was transferred from the EDTA-coated tube to a new microcentrifuge tube. Samples were then snap-frozen using liquid nitrogen and stored in a -80°C freezer, where they remained until time of processing and analysis for this study.

Of the 250 patients, an even number of treatment and control patients were selected to take part in this ancillary study. A total of 50 patients were selected (N = 25 treatment; N = 25 control) for all timepoints listed above (A, B, C, D). Chemiluminescence-based analysis and off-line analysis of plasma samples took place between January and March of 2025.

Free Hemoglobin, NO Consumption, and Clinical Data

This ancillary study is derived from a long-term clinical trial. As such, different data pertaining to this study have been gathered by members of the Berra group and stored in MGH-approved servers.

Patient demographics and laboratory values, collected at the time of the original study, and through the patient's electronic medical record, were stored and accessed via RedCap. Serum creatinine (mg/dL) and eGFR (mL/min/1.73m^2) are reported. eGFR was calculated through *https://qxmd.com/calculate/calculator_251/egfr-using-ckd-epi-2021-update*. Additionally, values pertaining to free hemoglobin and NO consumption data, previously measured by members of the Berra group through chemiluminescence assay, were stored via MGH Dropbox. Although free hemoglobin and NO consumption levels were already gathered by the Berra group, only original data and interpretations are included in this work. The appropriate 50 subjects in this ancillary study were the only ones whose data are included in this work. Appropriate data was identified, separated, and underwent statistical analysis. This data shows the degree of hemolytic activity within the samples present and aids in analyzing the role plasma nitrite and nitrate levels have in post-CPB AKI. Creation of graphs pertaining to stored data is described in the succeeding subsection.

Statistical Analysis

All 50 patients were evenly divided with respect to study group allocation. Data was collected and stored using Excel. Mean distribution was acquired using Excel and reported with +/- standard distributions (SD). For non-continuous categories (i.e. questionnaires), the sum total of each categorical selection was calculated along with the percentage pertaining to each specific group (treatment/control).

A two-way ANOVA and Tukey's multi-comparison test was performed on all nitrite, nitrate, free hemoglobin and NO consumption values through GraphPad Prism 10.4.0. This data was divided between control and treatment groups for comparison. A t-test was performed through GraphPad Prism for endpoint comparison between study groups and each timepoint. All figures, with the exception of Figure 1, were produced through the GraphPad Prism software. All tables were created using Microsoft Word software.

Demographic data was included for analysis. Average age with +/- SD was found, along with total and percentage data for race, ethnicity, and sexual demographics. This data was not separated based on study group. Body-Mass Index is also included but analyzed through the mean of the overall study subjects, with +/- SD included. Finally, total rates of AKI present within each group were calculated, along with the respective percentages per group and *p* values.

RESULTS

Of the 250 patients enrolled in the clinical trial, 50 were randomly chosen for this ancillary study, evenly distributed between the control and treatment groups. All 50 patients included in this study had confirmed degrees of endothelial dysfunction, as assessed by a screening questionnaire (Table 3). Demographic information, past medical history, and important hospital outcomes are presented in Tables 1, 2, and 4, respectively. Plasma samples from baseline, end of CPB, 24 hours post-CPB, and 48 hours post-CPB were analyzed for both control and treatment groups (Figure 2, 3). Creatinine and eGFR values are included for all patients to account for AKI development, as per KDIGO standards (Tables 5, 6). Degrees of AKI are illustrated in Table 7. Finally, free hemoglobin and NO consumption data were measured (Figure 4, 5).

Patient Population

Of the 50 patients chosen, males accounted for 41 (82%) of patients enrolled in this study. Average age of enrolled patients was found to be 65.06 years of age (+/- 8.69) with an average body mass index (BMI) of 29.40 (+/- 5.09). Although an even distribution between races would have been desired for the study, 48 of the 50 patients (96%) identified as white, while only 2 (4%) identified as Asian. Race was not a factor used when randomly enrolling patients (Table 1).

Total (N = 50)	
Subject Characteristics	
Age (years)	65.06 (8.69)
Male (No.)	41 (82%)
Female (No.)	9 (18%)
BMI (kg/m^2)	29.40 (5.09)
Race	
White	48 (96%)
Asian	2 (4%)

Table 1. Patients Enrolled Demographics for all patients enrolled in the study are shown. Age and BMI for all patients are shown as average and (+/- SD). Total number and (percent) of subjects is shown for sex and ethnicity. Patients are not divided based on study group. Abbreviations: BMI = Body Mass Index.

Patients enrolled in the treatment group had higher percentages of baseline chronic kidney disease, arrhythmias other than atrial fibrillation, and other non-listed medical conditions (12% *vs.* 4%; 8% *vs.* 4%; 44% *vs.* 20%). In contrast, those in the control group had higher rates of diabetes, prior heart surgery, obesity, chronic artery dieses, stroke and transient ischemic attack (28% *vs.* 12%; 12% *vs.* 4%; 32% *vs.* 12%; 8% *vs.* 4%; 8% *vs.* 0%). Hypertension, chronic heart failure, chronic artery disease, and hyperlipidemia were similar in percentage among both groups of patients (Table 2).

	NO **(N = 25)**	**N_2** **(N = 25)**
	Total (%)	**Total (%)**
Hypertension	17 (68)	20 (80)
Chronic Heart Failure	1 (4)	1 (4)
Diabetes	3 (12)	7 (28)
Chronic Kidney Disease	3 (12)	1 (4)
Prior Heart Surgery	1 (4)	3 (12)
Other	11 (44)	5 (20)
Arrythmia (besides atrial fibrillation)	2 (8)	1 (4)
Obesity	3 (12)	8 (32)
Chronic Artery Disease	10 (40)	9 (36)

Stroke	1 (4)	2 (8)
Transient Ischemic Attack	0 (0)	2 (8)
Hyperlipidemia	21 (84)	23 (92)

Table 2. Patient Past Medical History Relevant past medical history is shown. Patients are divided by study group and data is illustrated as total and (percent) per group. Abbreviations: NO = Nitric Oxide; N_2 = Dinitrogen Gas.

Regarding endothelial dysfunction screening, more treatment group patients were described as having higher percentages of hypercholesterolemia (40% *vs.* 24%). Control patients were found to have more active smokers (12% *vs.* 0%) and diabetic patients requiring oral hypoglycemic medication or were insulin-dependent (24% *vs.* 8%). Additional factors measured to gauge endothelial dysfunction at the time of screening are shown in Table 3.

	NO **(N = 25)**	**N_2** **(N = 25)**
	Total (%)	**Total (%)**
Coronary Artery Bypass Graft or Stent Placement	3 (12)	4 (16)

Intermittent claudication, critical limb ischemia or peripheral vascular disease (exception of vasculitis)	2 (8)	1 (4)
Transient ischemic attack	1 (4)	4 (16)
Diabetes requiring oral hypoglycemia agents or insulin	2 (8)	6 (24)
Hypercholesterolemia	10 (40)	6 (24)
BMI greater than 40	0 (0)	2 (8)
Hypertension and taking anti-hypertensive drugs	19 (76)	20 (80)
Active smoking ≥ 10 pack - years?	0 (0)	2 (12)

Table 3. Endothelial Dysfunction Screening Criteria Each potential patient was screened using a questionnaire to measure if they had endothelial dysfunction. If a patient was positive for at least one, they were eligible for enrollment. Patients may be positive for more than one comorbidity. Data is evenly divided between study groups, with 25 patients per group. All data is shown as a total per group and (percent per group). Abbreviations: NO = Nitric Oxide; N_2 = Dinitrogen Gas.

Hospital Course

No significant statistical difference was found between treatment and control groups regarding CPB duration (164.24 mins *vs.* 157.00 mins; *p* = 0.4093), time spent on mechanical ventilation (17.76 hrs *vs.* 16.19 hrs; *p* = 0.6591), intensive care unit (ICU) length of stay (LOS) (2.12 days *vs.* 2.71 days; *p* = 0.5017), or hospital LOS (10.04 days *vs.* 10.00 days; *p* = 0.9892). None of the 50 patients enrolled in this study died during hospital admission (Table 4).

	NO (N = 25)	**N_2 (N = 25)**	***P* Value**
CPB Duration (min)	164.24 (51.96)	157.00 (32.91)	0.4093
Time on Mechanical Ventilation (hrs)	17.76 (15.77)	16.19 (7.37)	0.6591
ICU LOS (days)	2.12 (1.72)	2.71 (4.00)	0.5017
Hospital LOS (day)	10.04 (11.61)	10.00 (8.91)	0.9892

Table 4. Hospital Outcomes Important data pertaining to patient hospital stay are shown. Patients are divided based on study group and presented as mean (+/-SD). *P* values per paired t-test for treatment vs. control groups. Abbreviation: NO = Nitric Oxide; N_2 = Dinitrogen Gas; CPB = Cardiopulmonary Bypass; ICU = Intensive Care Unit; LOS = Length of Stay.

Plasma Nitrite

Plasma samples from each group at all four timepoints were analyzed for all 50 patients. No statistical significance was noted between baseline control and treatment samples ($p = 0.4724$; Figure 2). For samples taken at the end of CPB and 24 hours after CPB, a significant difference was noted between the control and treatment samples (mean difference -0.6506 µM, 95% CI -0.8094 to -0.4918, $p < 0.0001$; mean difference -0.6183 µM, 95% CI -0.7771 to -0.4595, $p = 0.001$, respectively; Figure 2). Although not as drastic as the second and third timepoint, nitrite plasma samples from 48 hours after CPB did have a statistically significant difference between control and treatment patients (mean difference -0.1938 µM, CI 0.3526 to -0.033504, $p = 0.017$; Figure 2). *Post hoc* analysis from this data revealed considerable differences in plasma nitrite concentrations between baseline and the remaining three timepoints within the treatment group, but no such differences were noted in the control group.

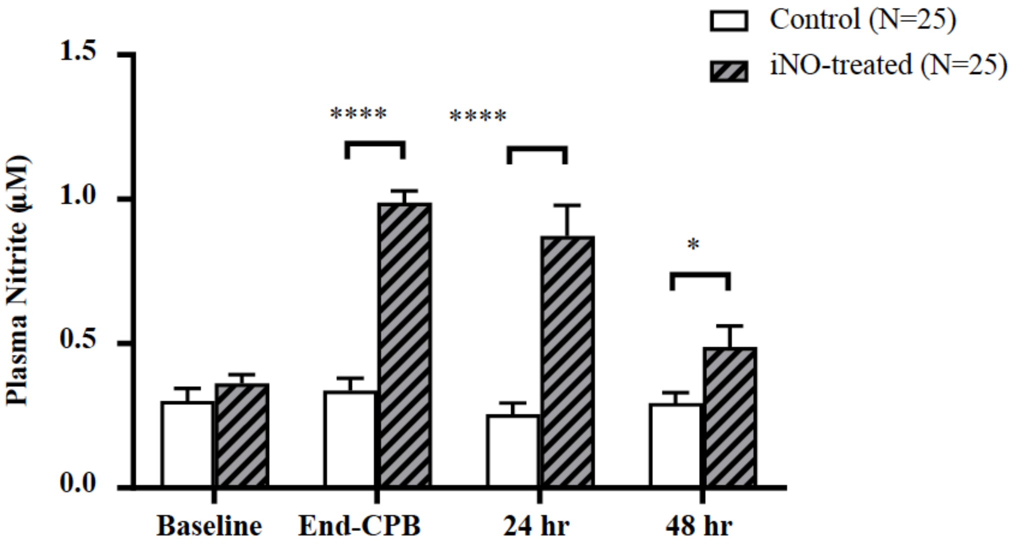

Figure 2. Plasma Nitrite Samples Concentrations All 50 plasma nitrite samples analyzed are illustrated and shown in µM concentration. *$p < 0.05$, ****$p < 0.0001$ by two-way ANOVA and Tukey's multi-comparison of control and treated patients. Abbreviations: iNO = Inhaled Nitric Oxide; CPB = Cardiopulmonary Bypass; Hr = Hour.

Plasma Nitrate

The same plasma samples used for nitrite analysis were used for plasma nitrate analysis. As with the nitrite samples, plasma nitrate between group and treatment patients did not significantly differ in baseline concentration ($p = 0.9538$; Figure 3). Control samples had a statistically significantly lower concentration compared to treatment groups at the end of CPB (mean difference -44.81 µM, 95% CI -84.07 to -5.559, $p = 0.0255$; Figure 3). Once in the CSICU, those treated with iNO for 24 hours had a significantly higher plasma nitrate concentration than those treated with the placebo (mean difference -168.7 µM, 95% CI -207.9 to -129.4, $p < 0.0001$; Figure 3). At 48 hours after CPB, treatment samples still showed a statistically significantly higher plasma nitrate concentration (mean difference -53.78 µM, 95% CI -93.03 to -14.52, $p = 0.0075$; Figure 3). Like the nitrite samples, *post hoc* analysis on plasma nitrate samples also showed a large and significant increase in plasma nitrate concentrations within the iNO group between baseline and the other three timepoints. Although there was an increase in plasma nitrate concentrations at the end of CPB, it did not appear to be as large of an increase as noted in the nitrite samples. Nitrate concentrations remained consistent for all four time points within the control group.

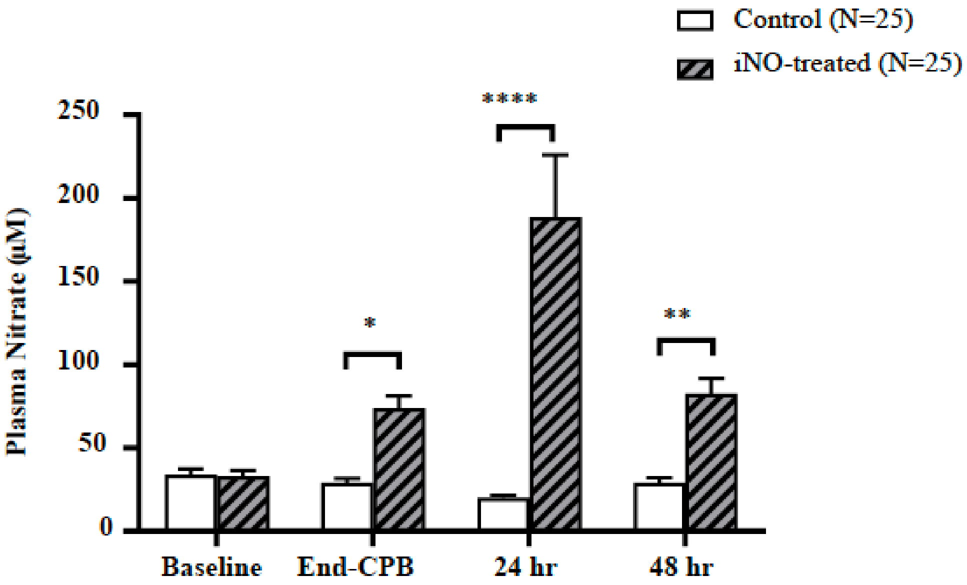

Figure 3. Plasma Nitrate Sample Concentrations All 50 nitrate sample concentrations analyzed are illustrated and shown in µM. *$p < 0.05$, ****$p < 0.0001$ by two-way ANOVA and Tukey's multi-comparison of control and treated patients. Abbreviations: iNO = Inhaled Nitric Oxide; CPB = Cardiopulmonary Bypass; Hr = Hour.

Free Hemoglobin and Nitric Oxide Consumption

Of the 250 patients enrolled in the clinical trial, the appropriate 50 patient values pertaining to free hemoglobin and NO consumption levels were located. The free hemoglobin values for 24 control patients could be located. All treatment patient-free hemoglobin values were properly located. Plasma-free hemoglobin was only statistically different, for both control and treatment groups, at the end of CPB ($p < 0.0001$; Figure 4), but not statistically different between the two groups. All other timepoints revealed consistent concentrations.

Plasma NO consumption data was located for only 16 and 22 of the control and treatment patients, respectively. Similar to the free hemoglobin samples, there was a significant increase in NO consumption at the end of CPB in the control group compared to the treatment group ($p < 0.0001$; Figure 5). No other differences between time points or groups were noted.

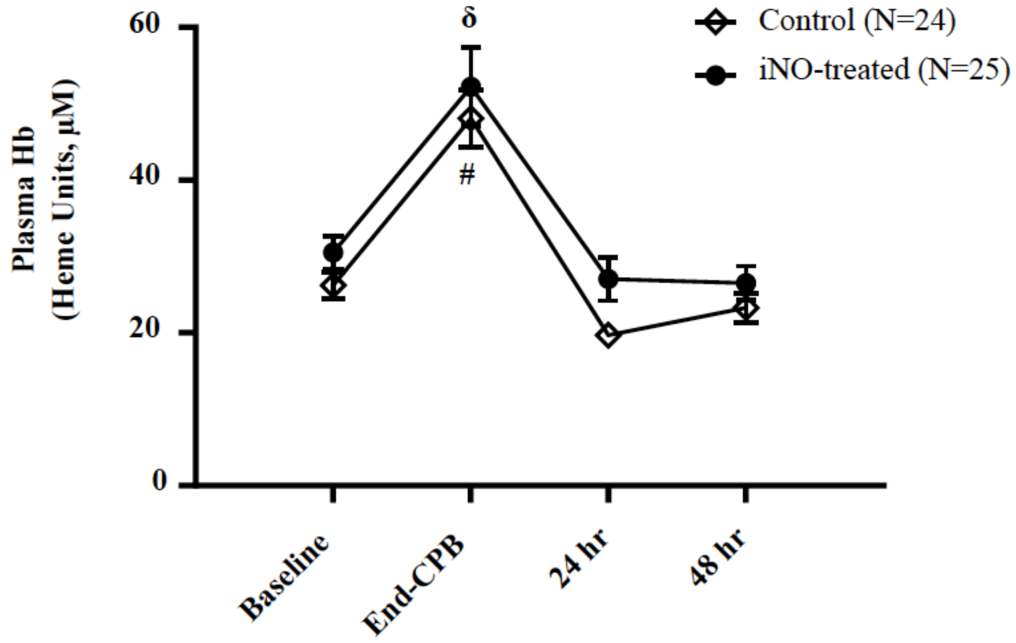

Figure 4. Free Hemoglobin Concentration Plasma free hemoglobin for plasma 24 control and 25 treatment plasma samples are shown in µM. #$p < 0.0001$; δ$p < 0.0001$ by two-way ANOVA and Tukey's multi-comparison of End-CPB and remaining three time points for control and treated patients, respectively. Abbreviations: Hb = Hemoglobin; iNO = Inhaled Nitric Oxide; Hr = Hour.

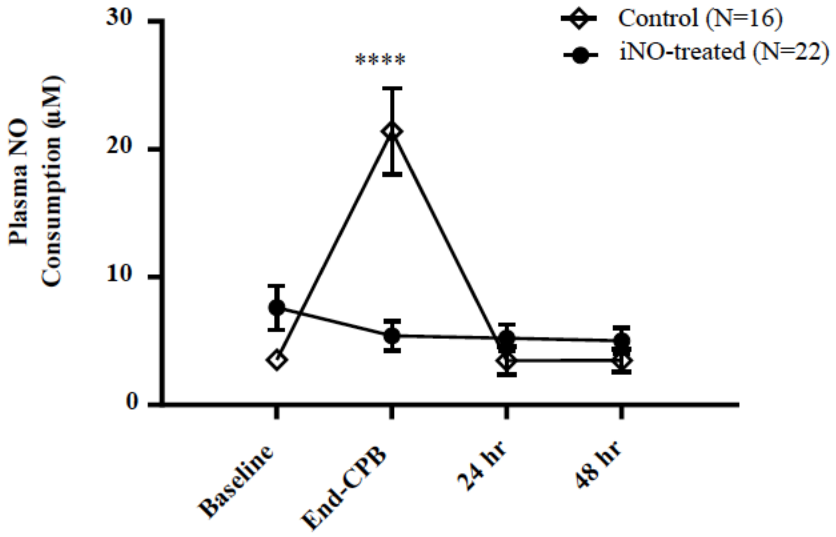

Figure 5. Plasma NO Consumption NO Consumption shown in µM. Data was only found for 16 control and 22 treatment groups. ****$p < 0.0001$ by two-way ANOVA and Tukey's multi-comparison of control and treated patients. Abbreviations: NO = Nitric Oxide; iNO = Inhaled Nitric Oxide; CPB = Cardiopulmonary Bypass.

AKI Outcomes

Laboratory values pertaining to AKI development per KDIGO guidelines were found for all 50 patients. Creatinine and eGFR values remained consistent between both study groups and did not produce statistically significant differences (Table 5 and 6, respectively). AKI rates between the study and the control group also yielded no statistical differences (Table 7). Of note, 3 patients enrolled in the treatment group developed stage 2 AKI while no control patients developed stage 2 AKI ($p = 0.0767$; Table 7). Additionally, no treatment patients developed stage 3 AKI, while 2 control patients did ($p = 0.1551$; Table 7).

Time Period	Group	Creatinine	*P* Value
Baseline	iNO	0.84 (0.22)	0.9154
	N_2	0.85 (0.10)	
15-minutes after CPB	iNO	0.85 (0.20)	0.7093
	N_2	0.87 (0.24)	
24 Hours after CPB	iNO	0.92 (0.31)	0.8682
	N_2	0.93 (0.26)	
48 Hours after CPB	iNO	1.07 (0.42)	0.6504
	N_2	1.02 (0.28)	

Table 5. Patient Creatinine Values Data presented for all enrolled patients, divided based on study group as mean (+/- SD). Serum creatinine values shown as mg/dL. *P* values per paired t-test for treatment vs. control group. Abbreviations: iNO = Inhaled Nitric Oxide; N_2 = Dinitrogen Gas; CPB = Cardiopulmonary Bypass

Time Period	Group	eGFR	*P* Value
Baseline	iNO	91.17 (15.57)	0.4343
	N_2	86.83 (8.98)	
15-minutes after CPB	iNO	88.88 (15.14)	0.1476
	N_2	80.52 (23.83)	
24 Hours after CPB	iNO	80.76 (22.28)	0.4279
	N_2	74.64 (18.97)	
48 Hours after CPB	iNO	77.08 (26.25)	0.7033
	N_2	74.65 (18.05)	

Table 6. Patient eGFR Values Data presented for all enrolled patients, divided based on study group, as mean (+/- SD). All eGFR values are as mL/min/1.73m^2 and were calculated through *https://qxmd.com/calculate/calculator_251/egfr-using-ckd-epi-2021-update*. *P* values per paired t-test for treatment vs. control group. Abbreviations: iNO = Inhaled Nitric Oxide; N_2 = Dinitrogen Gas; CPB = Cardiopulmonary Bypass; eGFR = Estimated Glomerular Filtration Rate.

	iNO (N = 25)	**N_2 (N = 25)**	***P* Value**
AKI	12 (48)	9 (36)	0.4004
Stage 1	9 (36)	7 (28)	0.5538
Stage 2	3 (12)	0 (0)	0.0767
Stage 3	0 (0)	2 (8)	0.1551

Table 7. AKI Across Study Groups Rates of AKI are shown as total and (percent per group). *P* values per paired t-test for treatment vs. control group. Abbreviation: AKI = Acute Kidney Injury; iNO = Inhaled Nitric Oxide; N_2 = Dinitrogen Gas.

DISCUSSION

The development of AKI after cardiac surgical procedures requiring the use of CPB has been listed as one of the most severe complications associated with CPB-based procedures. The original endpoint of this ancillary study was to measure the role of plasma nitrite and nitrate levels in cardiac surgical patients treated with iNO in hopes of preventing AKI. There were no differences in AKI, serum creatinine or eGFR levels between control and treatment patients ($p = 0.4004$; Table 7, 5, 6, respectively). Although AKI levels did not differ between the two groups, the substantial differences in plasma nitrite and nitrate levels (Figures 2 and 3, respectively) noted between both groups indicate the successful intervention through iNO during CPB.

Pathophysiology of iNO during CPB

Understanding the biochemical pathophysiology of what occurred during this study is important to explain why this study was successful despite the lack of statistical difference noted in AKI prevention.

Patients with confirmed endothelial dysfunction were enrolled in a clinical study with the aim of preventing the development of AKI following cardiac surgery due to CPB-associated hemolysis. It hypothesized that AKI could be prevented by administering 80 ppm of iNO through the CPB oxygenator during the procedure, then continuing through mechanical ventilation or HFNC once in the CSICU. During CPB, RBCs experience a high degree of hemolysis due to the velocity experienced by the cells traveling through the CPB circuit, the surgical procedure itself, and the use of different blood products throughout the case. This releases a significant amount of free hemoglobin during the procedure, acting as an NO scavenger, thus depleting levels of endogenous NO (Berra et al., 2012; Schaer et al., 2024). It has been well documented that by administering iNO during these types of procedures, you are now introducing an excess concentration of NO that can (1) bind with free hemoglobin in the CPB and thus preventing it from scavenging endogenously produced NO, and (2) providing an additional source of NO to further enhance the therapeutic qualities of the molecule. This is not a novel concept and has been well-documented through many clinical trials (Kamenshchikov et al., 2019, 2022; Lei et al., 2018).

Similar studies showed a successful reduction of AKI in enrolled patients (Kamenshchikov et al., 2022; Lei et al., 2018) and better outcomes related to cardiovascular protection (Kamenshchikov et al., 2019). Although they showed a positive correlation between the use of iNO and beneficial outcomes during these procedures, no study could definitively prove iNO's role. Yes, the biochemical properties of iNO should, in theory, work as a therapeutic tool to combat CPB-related hemolysis and should allow for reduction in reperfusion injury. However, this cannot be confirmed through detection of plasma NO concentrations due to the short half-life experienced by the molecule (Kelm, 1999). Although briefly mentioned in one study, the only way to adequately confirm successful administration of iNO is through analysis of its metabolic byproducts (Kamenshchikov et al., 2019; Lauer et al., 2001).

Plasma Samples Analysis

Of the 50 patients enrolled in this ancillary study, 25 belonged to the treatment group. Every patient in the treatment group experienced a substantial increase in plasma nitrite and nitrate concentration after iNO administration. Given that nitrite and nitrate are the oxidized metabolic products of NO, this does not come as a surprise. Such significant increases in plasma nitrite and nitrate concentration indicate successful administration of iNO and show that administered NO was indeed metabolized into products that increase its systemic bioavailability.

An increase in plasma nitrite concentrations in treated patients after CPB and 24 hours post-procedure, as noted in Figure 3, is consistent with the metabolic properties of NO. Compared to plasma nitrate levels of treated patients, plasma nitrite decreased at the 48-hour mark and was closer to the concentration in the control group. This is likely because of the oxidation from nitrite to nitrate and the longer half-life exhibited by plasma nitrate. Additionally, plasma nitrate concentrations of treated patients do not show as substantial of an increase after CPB as seen with plasma nitrite, which again confirms NO's metabolic properties.

Free hemoglobin levels were analyzed to measure the degree of hemolytic activity (Figure 4). To no surprise, both groups of patients showed a sharp increase in hemolytic activity immediately after CPB ($p < 0.0001$), which was expected as this is incredibly traumatic for the RBCs. Differences in hemolytic activity were not expected to differ in the groups, as the physical procedure is still the same for both groups. NO consumption was also measured and showed increased NO consumption after CPB in control patients

($p < 0.0001$), indicating an increase in free hemoglobin NO scavenging activity (Figure 5). After CPB, NO consumption rates in control patients returned to baseline, which can be explained by a decrease in free-floating hemoglobin once the traumatic procedure has concluded. Interestingly, treated patients did not show an increase in NO consumption, meaning that administration of iNO was successful in preventing the damaging effects of free hemoglobin and serve to further confirm successful administration of intervention. However, it is vital to note that the NO consumption observed in these samples is almost less than half of those observed in Lei's study population during the same timepoints. This likely illustrates that the Chinese population experienced more significant instances of hemolysis during CPB (Lei et al., 2018). This could explain why, even though there is evidence of successful iNO administration, there was a failure in the ultimate end goal of this study in reducing AKI. Simply put, if there is no injury then there is no injury to fix.

Importance of Increased Plasma Nitrite and Nitrate Levels following iNO Therapy

As stated in the preceding subsection, increased plasma nitrite and nitrate concentrations prove successful administration of iNO. More importantly, however, it may serve as a way to improve the bioavailability of NO throughout the body. As Nagasaka and colleagues demonstrated, plasma nitrite levels metabolized from iNO administration can be converted back to NO, thereby enhancing the bioavailability of this unstable signaling molecule and functioning as a feedback loop (Nagasaka et al., 2008). Given the short half-life of NO, it is likely to metabolize quickly into nitrite once in circulation. As such, a possible mechanism for the positive effects observed with iNO administration can be attributed to the increased bioavailability of NO through its metabolites. This concept is similar in theory to the administration of nitrate derived drugs, such as nitroglycerine (Levy, 2005). Additionally, the existence of the non-canonical pathway shows that the reduction of NO metabolites into NO is possible and something which the body does regularly (Lundberg et al., 2008; Weitzberg et al., 2010).

The failure of the NITRIC trial, published in 2022, serves as an example of why this mechanism works. The NITRIC trial, consisting of 1371 infants, aimed to reduce the amount of time infants spent on a ventilator following CPB. This was attempted through the administration of 20 ppm of iNO during CPB (Schlapbach et al., 2022). Although a similar successful trial was used as a proof of concept (Checchia et al., 2013), the NITRIC trial was unsuccessful. None of the patients treated with iNO experienced the positive benefits of iNO, completely contradicting any of the literature on the topic. An important aspect of the NITRIC trial was the low dose of 20 ppm of iNO administered to patients only while on CPB. Additionally, the NITRIC trial failed to show if any NO metabolites were present in plasma samples from patients treated with iNO. As a prior listed CPB-NO trial, NO metabolites are indicators of successful iNO administration (Kamenshchikov et al., 2019). The NITRIC trial's failure is likely a result of the severely hemolytic environment that exists within the CPB circuit, meaning the iNO administered would have been consumed by the free hemoglobin prior to it even making it to circulation. Furthermore, met-Hb levels in treated patients only reached 0.4%, a significantly low number when giving iNO, signifying the probability of not enough NO being delivered. Similar concentrations of met-Hb were noted in our study. Had an increased concentration of iNO been given, it would likely not have been affected by the hemolytic environment, which would have been confirmed if NO metabolites had been measured. A higher dose of iNO would have produced sufficient NO metabolites to deliver NO systemically prior to its consumption.

In contrast to the NITRIC study, the previously mentioned Nitrate-CIN trial was successful. This study takes advantage of the non-canonical pathway for NO production by administering oral nitrate salt tablets (Jones et al., 2024). Jones and colleagues successfully prevented kidney injury after administering contrast dye, which raises hemolysis rates and exhibits nephrotoxic effects similar to CPB-acquired hemolysis. Using nitrate salt tablets, the Jones team increased plasma nitrate and nitrite levels to colossal levels, successfully allowing for XOR and other nitrite reductases to reduce the compounds into NO. This effectively recreated the same nephroprotective effects of iNO through the non-canonical pathway and emphasizes the growing relevance of plasma nitrite and nitrate levels, as it demonstrates that these byproducts indeed enhance the bioavailability of NO, which can be manipulated for therapeutic purposes.

Although plasma nitrite and nitrate concentrations were significantly increased after iNO administration in the treatment group for this study, it is nowhere as drastic as an increase as that noted in the Nitrate-CIN trial. The concentrations noted by Jones were significantly higher and explain why their study was successful. Their nitrate salt administration allowed NO to be created through the non-canonical pathway. In contrast, iNO administration through CPB, as done in this study, could not produce similar levels of NO metabolites, possibly preventing a “therapeutic” threshold to be reached. Finally, in addition to have similar levels as the Nitrite-CIN trial, significantly lower levels of hemolysis in our patient population could have been an additional factor that resulted in negative results.

Limitations

As previously stated, this study aimed to show how increased plasma nitrite and nitrate levels after iNO administration during CPB would successfully prevent AKI. That did not occur and goes against published literature. A prior clinical trial using a similar methodology, but in healthy Chinese patients, was successful in preventing AKI in roughly 30% of those treated with iNO during CPB (Lei et al., 2018). Considering we used similar methods but in a population with confirmed endothelial dysfunction, which has been shown to benefit from iNO administration during severe hemolysis (Berra et al., 2014), it is surprising to observe almost equal rates of AKI between the control and treatment groups. Plasma nitrite and nitrate concentrations, as well as rates of NO consumption and plasma-free hemoglobin, prove that iNO was correctly administered, entered systemic circulation, and effectively acted on its desired target. It is possible that patients enrolled in the clinical trial simply did not experience as severe levels of hemolysis as those enrolled in different clinical trials, the difference of almost double the amounts of NO consumption between Lei's patients and those in this study suggests this. This could potentially be explained by the presence of hemolytic filters built into the CPB device. Our population also did not experience the same degree of plasma nitrite and nitrate concentration increase as other studies listed previously in this work. It should also be noted that high dose iNO (160-300 ppm) could serve as a possible alternative method for therapeutic effect. Another limitation of this study is that all work occurred in one medical center; thus, it is possible that clinical standards apart from our medical facility could have unknowingly affected the results of this study. It would be interesting to re-analyze this data but with the additive factor of accounting for different severities of hemolysis experienced by patients. Finally, only 1/5 of patients enrolled in the clinical trial were enrolled in this ancillary study, and thus it may be premature to make such conclusions using only 1/5 of available samples.

Final Remarks

The therapeutic effects of iNO for CPB-associated hemolysis were unfortunately not seen in the 50 patients enrolled in this ancillary study. The two study groups had no statistical differences in serum creatinine, eGFR, or AKI rates. Significant differences were noted in plasma nitrite and nitrate concentrations between treated and non-treated patients, added with differences in NO consumption, prove that iNO was successfully administered and delivered systemically. This sheds light into the possible mechanism of NO delivery throughout the body after iNO administration. Further research should focus on further exploiting plasma nitrite and nitrate levels and measuring NO activity following manipulation.

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CURRICULUM VITAE

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