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Design of a bioreactor to study the role of red blood cells in the transport of nitric oxide in the microcirculation

Nupura Bhise^{} and Mahendra Kavdia[†]*

ABSTRACT

Nitric oxide (NO) plays an important role in physiological functions like vasodilation, neurotransmission, and inhibition of platelet aggregation. The endothelium-derived NO diffuses into the vascular lumen where it interacts with flowing blood as well as the smooth muscles where it modulates vascular tone. However, uncertainty exists on how NO escapes the rapid scavenging by hemoglobin (Hb) and reaches smooth muscles. Several proposed hypotheses include 1) a reduced reaction rate of NO with Hb contained inside red blood cells (RBCs) and 2) NO preservation in the bound form of s-nitrosohemoglobin or nitrite. The mechanism and magnitude of reduction of NO reaction rate with Hb contained inside RBCs are not well established. In this study, an in vitro experimental system was designed to expose stirred RBC suspension to physiologically relevant NO flux. NO-RBC interactions were studied by measuring the reaction products, nitrite and total NO_x, using chemiluminescence method. We studied the effect of increasing hematocrit from 5% to 45% on NO-RBC interaction under oxygenated condition. Results show that the system maintained a steady state in the bioreactor and could be easily modified to control NO delivery flux. An increase in product concentration was observed by increasing the hematocrit from 5% to 45%. The study is clinically important as the understanding of molecular interaction of NO with Hb in RBCs and mode of NO transport in microcirculation may provide therapeutic opportunities in the biomedical field in areas as diverse as sickle cell anemia, septic shock, hypoxic pulmonary vasoconstriction, and blood substitutes.

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INTRODUCTION

Nitric oxide (NO) mediates significant vascular endothelium functions, including vasodilation, blood pressure regulation, inhibition of platelet aggregation, neurotransmission, and immunological response (Moncada et al., 1991). In the microcirculation, NO is synthesized via enzymatic oxidation of amino acid L-arginine to L-citrulline by endothelial NO synthase enzyme. Endothelium-derived NO diffuses into the vascular lumen where it interacts with flowing blood as well as smooth muscles where it activates soluble guanylate cyclase and modulates vascular tone. Discovery of NO as endothelium-derived relaxation factor led to the awarding of a 1998 Nobel Prize in physiology to investigators F. Murad, R.F. Furchgott, and L.J. Ignarro (Gow et al., 1999). Subsequently many scientific investigators have been involved in studying a possible role for red blood

cells (RBCs) in transport of NO and its vascular effect in facilitating flow of blood through the microcirculation.

Homeostasis of NO is a balance between its synthesis and consumption. In the lumen, NO is either oxidized by oxygen (O_2) resulting in nitrite (NO_2^-) formation, or is taken up by red blood cells, which contain high concentrations of hemoglobin (Hb), an effective NO scavenger. Two important reactions of Hb and NO considered to be relevant are (a) the oxidation, in which NO reacts with oxyHb ($HbFe^{2+}O_2$) to form nitrate and metHb ($HbFe^{3+}$), and (b) the addition reaction, in which deoxyHb ($HbFe^{2+}$) binds to NO to form $HbFe^{2+}NO$ (HbNO). Due to this high reactivity of NO with Hb and other species, NO may be consumed before it travels from its synthesis site (endothelium) to target site (smooth muscle). Earlier studies thus assumed that the only effect of RBCs on the bioactivity of NO was to scavenge and inactivate NO, thus limiting its ability for

MEET THE STUDENT-AUTHOR

I am an international student and graduated in spring 2007 with a biological engineering major. I completed high school in Mumbai, India, and joined the University of Arkansas in fall 2003. Beginning in November 2003, I worked as an undergraduate assistant for Dr. Mahendra Kavdia and helped him set up his Vascular Biotransport Laboratory; because of this opportunity, I fulfilled my professional aspiration to work in a biomedical laboratory setting under his guidance. I have been involved in research studying the in-vivo interaction of

red-blood cells with nitric oxide. My work, which was funded thrice by the U of A Honors College Undergraduate Research Grant, has yielded four conference presentations, and I plan to submit a paper for professional journal publication.

Since my freshman year at the university, I have been involved each year with on-campus professional and community organizations. I am currently serving as the president of the Biological Engineering Students Club and served as its treasurer in my junior year. I was also the president of the Golden Key International Honour Society and served as a mentor for the Society for Women Engineers. I have been awarded many prestigious academic scholarships and honors including the Outstanding Senior Scholar award (Arkansas Academy of Biological and Agricultural Engineers), and First-Ranked Senior Scholar, U of A. I have been accepted for the PhD program in biomedical engineering at the Johns Hopkins University and plan to begin in fall 2007.

I would like to thank Dr. Kavdia for his support and guidance in my research as well as academic career at the U of A.



Nupura Bhise

vasodilation (Nagababu et al., 2003). However, the NO bioactivity is preserved in the blood under normal physiological conditions. Scientists trying to investigate how NO escapes rapid scavenging by Hb have proposed various hypotheses including (a) an erythrocyte-free layer in the lumen adjacent to the endothelium that provides an effective diffusional barrier; (b) about a 500- to 1000-times lower effective reaction rate of NO with Hb contained in RBC than that with free Hb, $k \approx 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Huang et al., 2001; Liu et al., 2002); and (c) NO preservation in the bound form of s-nitrosohemoglobin (sNOHb) (Gow et al., 1999; Pawloski et al., 2001; McMahon et al., 2002).

In spite of these studies, individual roles of these proposed factors for regulation of vascular functions remain largely unknown. Uncertainty also exists on the mechanism responsible for NO preservation and the magnitude of effective reaction rate of NO with RBCs and its relationship with hematocrit and flow. To overcome the technical difficulties of in vivo determination of the local availability of NO and study this relationship, we designed an in vitro constantly stirred experimental setup that simulates in vivo conditions of NO synthesis and consumption. We delivered NO at a constant flux to a constantly stirred RBC solution in the bioreactor and measured the products of RBC-NO interaction: nitrite (NO_2^-) and total NO-species (NO_x), using a gas-phase chemiluminescence method.

The in vitro studies provide a critical step in quantifying NO uptake by varying the hematocrit concentration from 5% to 45% in oxygenated condition and extend our understanding of the functional role of NO in the microcirculation. This knowledge is critical to development of hemoglobin-based oxygen carriers (HBOCs) that are under development as an alternative to RBC transfusion because of the potential advantages of unlimited supply, no cross-matching requirement, chemical purity and prolonged storage (Winslow, 2000). The understanding of molecular interaction of NO with Hb and NO transport is clinically important and may provide therapeutic opportunities in areas as diverse as sickle cell anemia, septic shock, NO inhalation, hypoxic pulmonary vasoconstriction, and blood substitutes (HBOCs).

MATERIALS AND METHODS

Materials.

All the chemicals—sodium iodide (NaI), sodium nitrate (NaNO_3), sodium nitrite (NaNO_2), phosphate saline buffer (PBS), EDTA, vanadium (III) chloride (VCl_3), sodium hydroxide (NaOH), and free Hb—were American Chemical Society (ACS) grade and purchased

from Sigma–Aldrich (St Louis, Mo.). Glacial acetic acid (ACS grade) and 12 M hydrochloric acid (HCl, ACS grade) were obtained from VWR International (Fayetteville, Ar.). Thereafter, 1-mM stock solutions of NaNO_2 and NaNO_3 were prepared in de-ionized (DI) water and 4 mM PBS were prepared by dissolving one 10X PBS packet in 1000 ml DI water with 2 g of glucose added to it. Saturated VCl_3 solution was prepared by dissolving 0.8 g of VCl_3 in 100 ml of 1 M HCl and DI water and a 0.2 M solution of NaI was prepared by dissolving 0.29978 g of NaI in de-ionized water.

Design of an in vitro experimental system for RBC-NO interaction studies.

In this study, a continuously stirred novel bioreactor device was designed that replicated in vivo conditions of NO generation and consumption in blood vessels. All the reactions were conducted using this experimental system (figures 1A and 1B). A 100-ml Pyrex bottle was used as the bioreactor with a controlled headspace and stirring device for the experimental solutions. The headspace cover included two openings (to serve as inlet and outlet for gaseous mixture) and a third sample collection port. These openings consisted of Swage-lock fittings glued into holes drilled in the bottle cover. Controlled gas-flow meters (Porter Instrument Co. Hatfield, Pa.) were used to balance the gas mixture and obtain a specified NO concentration from gas cylinders entering the bioreactor, where NO reacted with 10 ml of treatment solution. Flow rate of gas mixture into the headspace consisted of 100 ml/min of 100% ultra-high pure nitrogen and desired ml/min of 10% NO/N_2 . The gas mixture was allowed to flow across stainless steel tubing to the inlet of the bioreactor in the chemical safety hood. The solution was continuously stirred with a magnetic stirrer at a constant speed of 2 rpm. To characterize the bioreactor in terms of NO delivery, we treated 10 ml of DI water at two different NO fluxes, and 100 μl samples were collected at 1, 2, 3, 4, 5, and 10 min. Nitrite concentration was measured in collected samples using chemiluminescence method. Next we conducted a variable-time experiment with 5% oxygenated RBC solution and measured both the nitrite and total NO_x concentrations. The ability of the device to study NO-RBC interactions was validated by treating oxygenated RBC solution at 5% and 45% hematocrit and measuring the respective reaction products.

Preparation and treatment of 5% and 45% RBC solutions.

Approximately 100 ml of pig blood were collected from the Savoy Experimental Farm, University of Arkansas, in 10-ml heparinized tubes (Vacutainer). Blood was centrifuged at $\text{RCF} = 800\text{g}$, $\text{RCM} = 2713$, $\text{Time} = 10 \text{ min}$ and $\text{Temperature} = 4^\circ\text{C}$. Plasma was pipetted out and RBCs were washed thrice with 2-3 mL refrigerated PBS solution

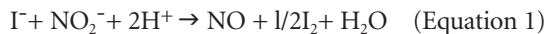
(4 mM PBS + 2 g glucose). After separation of RBCs, the RBC/PBS solution was stored at 4°C to prepare the hematocrit solutions. Thereafter, 1 ml of RBC, obtained by centrifugation of pig blood, was mixed with 19 ml of prepared PBS solution to prepare 20 ml of 5% RBC solution, and 9 ml of RBC were mixed with 11 ml of PBS solution to obtain 20 ml of 45% RBC solution. For both the variable-time and variable-hematocrit experiments, 10 ml of the prepared RBC solution were treated for 10 minutes in the bioreactor under oxygenated conditions. Gas flow rates were adjusted at 100 ml/min of 100% N₂ and 0.7 ml/min of 10% NO/N₂ mixture, and the solution was constantly stirred.

Collection of samples.

For variable-time experiments, about 0.3 ml of blood samples were collected with a 1-ml syringe into eppendorf tubes at regular intervals of 2 min from the instant the NO/N₂ treatment (0-min blank sample) started till the end of the treatment (10-min sample). For variable-hematocrit experiments, only the blank blood sample (0 min) and the end sample (10 min) were collected. Then 1 μM, 5 μM, 10 μM, 50 μM, and 100 μM concentrations of NaNO₂ and NaNO₃ standard samples were prepared from the respective 1-mM stock solutions. The NaNO₂ and NaNO₃ standard curves were used to quantify the products NO₂⁻ and total NO_x, respectively.

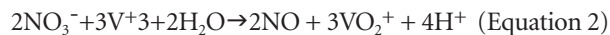
Chemiluminescence method for measurement of reaction products.

The Model 280i Nitric Oxide Analyzer (NOA) from Sievers Instruments (Boulder, Colo.), a high-sensitivity detector with a detection limit of 1 picomole, was used for measuring NO based on a gas-phase chemiluminescent reaction between NO and ozone. The purge-vessel setup was used to detect NO₂⁻ and a gas-bubbler NaOH trap was added to the setup to measure total NO_x. Argon that bubbled through the purge vessel carried any released NO to the NO detector. NO reacts with dissolved oxygen to form NO₂⁻. To measure NO₂⁻, the purge vessel was filled with a reducing agent (1% wt/vol of NaI or KI in glacial acetic acid) to convert NO₂⁻ to NO, which was then carried to the NOA for detection. The reaction converting NO₂⁻ to NO,



For most applications, ~8 ml (2 ml NaI and 6 ml acetic acid) of the reducing agent were prepared in the purge vessel, as this volume was sufficient for measurement of 20-50 samples, depending on the volume of the sample injected. The reagent was changed when the solution turned yellow due to formation of I₃⁻, indicating depletion of the reducing agent.

NO reacts with oxyHb to form NO₃⁻. To measure total NO_x, about 5 ml of vanadium (III) chloride in hydrochloric acid were used as the reducing agent to convert NO₃⁻ to NO,



To avoid foaming of the blood samples, 100 μl of antifoaming agent were injected into the reducing agent. To achieve high conversion efficiency, the reduction was performed at ~90°C.

For volumes corresponding to 100 μl of standard samples, 10 μl of blood samples for NO₂⁻ and 5 μl of blood samples for total NO_x were injected through a septum into the purge vessel to obtain concentration-dependent chemiluminescent signals in millivolts (mV). A calibration curve was prepared by plotting concentrations of standard solutions versus peak mV signals.

Statistics.

The DI-water experiments were conducted twice, changing the NO delivery rate. The experiments for 5% and 45% hematocrit were repeated three times under oxygenated conditions. The results presented were in the form of mean ± SE.

RESULTS AND DISCUSSION

Experimental system design.

The experimental system developed in the study was simple: to fabricate and reliably replicate the in vivo conditions of NO generation and consumption in blood vessels where flowing blood receives a constant NO flux from the endothelium, which forms the first layer of the vascular wall. The solution could be treated with a desired NO/N₂ flux for the complete duration of the experiment using the digitally controlled mass-flow meter and the setup could be modified to vary the NO+N₂ delivery rate into the solution in the bioreactor. The sample collection port on top facilitated a convenient withdrawal of sample from the solution without significant disturbance to the experimental conditions. The gas outlet port prevented buildup of gases in the bioreactor. Continuous stirring of the solution minimized effects of any effective diffusional barrier due to the unstirred layer surrounding the RBC, which is responsible for preserving NO bioactivity (Liu et al., 2002).

So far the majority of studies have been performed using NO-delivery systems like direct injection of saturated solutions of NO (Han et al., 2002; Joshi et al., 2002; Han et al., 2004) and NO donors (Vaughn et al., 2000). Disadvantages of using these direct-delivery systems are that they can cause significant loss of NO from the exper-

imental system (Kavdia and Lewis, 2003) and create high, localized concentrations of NO that react instantaneously before reaching uniform concentration in the system. They also contain nitrite as an impurity, which can affect the interpretation of RBC-NO interaction products (Nagababu et al., 2003). This in vitro, controlled, gaseous NO-delivery system helped to overcome technical difficulties involved in in vivo determination of local availability of the gas, maintained a homogeneous concentration of NO in the reaction environment, and also eliminated the possibility of product contamination. The system thus allowed RBC-NO reactions to take place as a steady state was maintained on both the liquid side and gaseous side, which more accurately simulated the physiological conditions in microcirculation.

Nitrite and total NO_x standard curves to quantify the treated samples.

Standard samples of NO₂⁻ and NO₃⁻ were injected into the NO analyzer to generate standard curves for both nitrite and total NO_x concentrations, respectively. Fig. 2A shows the chemiluminescent signal (mV) recorded by the NO analyzer after injecting standard nitrite samples in concentrations of 1, 5, 10, and 50 μM. Fig. 2B shows the peak mV value for each concentration of standard nitrite sample. Similarly, figures 3A and 3B show the chemiluminescent signal and peak mV signal, respectively, for total NO_x concentrations of 1, 5, 10, 50, and 100 μM.

The peak mV signals showed a linearly increasing trend with increases in nitrite and total NO_x concentrations. The standard curve data established a linear relationship between the standard concentration and peak mV signal. Thereafter, before injecting treated samples, a 1-μM standard sample was injected every time the reducing agent in the purge vessel was changed. The concentration of the treated sample was calculated using the formula,

$$\frac{[\text{Sample peak signal (mV)} - \text{Sample base signal (mV)}]}{[\text{Standard peak signal (mV)} - \text{Standard base signal (mV)}]} \times \text{Dilution factor}$$

(Equation 3)

where the base signal was the lowest value recorded before injecting the sample or standard and the peak value was the highest mV signal obtained after injecting the sample or standard.

DI water experiments to characterize the NO-delivery rate of the experimental system.

To characterize the experimental system, we conducted experiments by treating 10 ml DI water in the bioreactor and studied the effect of changing the gaseous partial pressure in headspace on the total NO-delivery rate (moles/min). Fig. 4A shows the NOA signal recorded for the nitrite analysis of DI water samples collected at reac-

tion times 0, 2, 4, 6, 8, and 10 min, when treated with a flow rate of 0.7 ml of 10% NO/N₂ + 100 ml of 100% N₂. Fig. 4B shows the nitrite concentration profile for DI water samples collected at regular reaction-time intervals. Similarly, figures 5A and 5B show the NOA signal and nitrite-concentration profile for DI water samples collected at reaction times 0, 1, 2, 3, 4, 5, and 10 min, conducted at flow rates of 1.5 ml of 10% NO/N₂ + 100 ml of 100% N₂.

From the best-fit trend-line (figures 4B and 5B), we noted a linear increase in nitrite concentration with each successive time sample injected from 0 min to 10 min for both cases. Slope obtained from the best-fit linear trend-line gave the delivery rate of NO in units of μM/min. NO delivery rate was converted to moles/min using the relationship,

$$\text{moles} = \text{concentration} \times \text{volume} \quad (\text{Equation 4})$$

where the volume was 10 ml. NO-delivery rates calculated using Equation 4 were 3.853 nmoles/min for a flow rate of 0.7 ml of 10% NO/N₂ + 100 ml of 100% N₂, and 22.22 nmoles/min for a flow rate of 1.5 ml of 10% NO/N₂ + 100 ml of 100% N₂.

The results indicated that the NO-delivery rate increased about 5.77 times as the 10% NO+N₂ flow rate increased from 0.7 ml/min to 1.5 ml/min. Nitrite concentration was time-dependent. With an increase in time during which the DI water solution was allowed to react with NO/N₂ gaseous mixture, there was a linear increase in the nitrite concentration in the solution. The NO reacts with dissolved O₂ to form nitrite, with a reaction rate $K_{\text{NO}^2\text{C}_{\text{O}_2}}$, where $K = 9.8 \times 10^6 \text{ m}^{-2}\text{s}^{-1}$. This implied that the experimental system was able to maintain a constant NO-delivery rate in the solution during the entire duration of the treatment and NO was allowed to mix homogeneously throughout the stirred solution. Thus our system has an advantage over systems in which addition of NO bolus causes rapid initial reaction before NO is homogeneously mixed throughout the solution (Joshi et al., 2002). The results proved that this in vitro system can be used to treat RBC solution with a constant NO flux, thereby more accurately replicating in vivo conditions of RBC-NO interactions. Thus using these preliminary data of nitrite formation with DI water, we were able to characterize the experimental system.

Nitrite and total NO_x levels in 5% oxyRBC solution.

Next, we treated 10 ml of 5% oxy RBC solution with 0.7 ml of 10% NO/N₂ + 100 ml of 100% N₂ for 10 min, regularly collecting samples at reaction times of 0, 2, 4, 6, 8, and 10 min. Figures 6A and 6B show the nitrite- and total NO_x-concentration profiles, respectively, for these

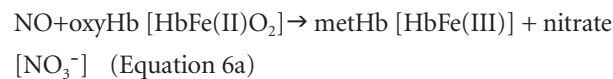
samples. The results showed that as treatment time increased from 2 min to 10 min, concentrations increased from 1.23 μM to 2.79 μM for nitrite and 10.3 μM to 25.1 μM for total NO_x . There was a linear increase in nitrite and total NO_x concentrations as reaction time between 5% oxy RBC and NO increased. Figure 6C shows percent ratio of nitrite to total NO_x in the 5% oxy RBC samples at different time points. The ratio remained almost constant at $11.87 \pm 0.876\%$ for 2-10 min.

Results indicate that our system maintained a steady-state at both gaseous and liquid levels in the bioreactor. Oxygenated RBCs rapidly scavenged NO at an uptake rate of 21.9 nmoles/min calculated from the total NO_x linear-concentration profile. Also nitrite was formed as a relatively minor portion of the total NO_x species, suggesting that the major part of the reaction products formed were either in the stable nitrate form or other intermediate species like S-nitrosithiols. Although nitrite is the main product of NO oxidation in blood plasma (equation 5), upon interaction with oxygenated heme domains in RBC, however, NO is rapidly oxidized to form nitrate and metHb (equation 6a). Our experiments suggest that the oxidation product nitrate is formed at a higher concentration than the conservation product nitrite. Similar observations were reported by Liao and colleagues, who conducted experiments by generating NO homogeneously using NO donors (Joshi et al., 2002). Thus nitrite and other NO-species like SNOHb, a product of s-nitrosation (Equation 6b), and HbFe(II)NO, a product of reductive nitrosylation (equations 6c and 6d), may be formed that contribute to the conservation of NO bioavailability in the microcirculation (McMahon et al., 2002), but the consumption reaction dominates the RBC-NO interaction in physiological conditions.

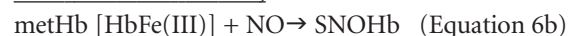
Oxidation of NO,



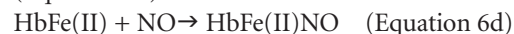
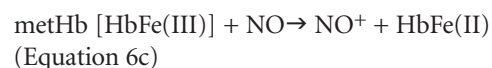
Reaction of NO with oxyRBC,



S-nitrosation of metHb,



Reductive nitrosylation of metHb,



Effect of variable hematocrit on nitrite and total NO_x levels in oxygenated RBC solution.

Products of the NO reaction with the RBC-encapsulated Hb depend on the concentration of the hematocrit and to study this effect, we conducted experiments by varying hematocrit from both 5% to 45% in oxygenated conditions. Three separate sets of experiments were conducted and mean values were plotted with standard error bars. Figures 7A and 7B show the comparison of nitrite and total NO_x concentrations, respectively, for samples collected at 10 min for each hematocrit percentage. The mean nitrite concentrations increased from $3.73 \pm 1.17 \mu\text{M}$ for 5% hematocrit to $5.61 \pm 3.73 \mu\text{M}$ for 45% hematocrit. Total NO_x concentrations also increased from $34.4 \pm 10.82 \mu\text{M}$ for 5% to $40.2 \pm 7.68 \mu\text{M}$ for 45% hematocrit. Mean nitrite concentration increased 1.5 times and total NO_x concentration increased 1.17 times as the hematocrit was increased from 5% to near physiological range of 45%. Figure 7C shows the comparison of nitrite to total NO_x -percent ratio for the two oxy RBC solutions. The ratio also increased from $10.84 \pm 4.28\%$ for 5% hematocrit to $13.96 \pm 10.93\%$ for 45% hematocrit solution.

As NO reacts homogeneously with increased concentration of RBC in the solution, there is an increased scavenging of NO by RBC-encapsulated Hb, thereby increasing the concentration of total NO_x (Azarov et al., 2005). Nitrite is formed as a product of oxygenation reaction of NO with O_2 in solution (equation 5) before NO can be rapidly scavenged by RBC-encapsulated Hb. Availability of higher levels of O_2 in oxygenated condition and more O_2 -bound heme Fe (II) sites to react with NO at higher hematocrit results in an increase of the nitrite concentration in oxygenated solution. Nitrite was formed in the range of 11 to 13% of the total NO_x species. Formation of the conservation product, nitrite, as a minor species indicates that NO and oxyRBC interaction is dominated by the consumption reaction to form metHb and nitrate (equation 6a). The dominance of the consumption pathway was observed by Gladwin and colleagues, and also in experiments conducted by Liao and colleagues using NO donors (Gladwin et al., 2000; Han et al., 2004). Liao and colleagues further reported that in experiments conducted using saturated NO bolus, intermediate species like SNOHb and HbFe(II)NO were formed as minor portions of the total NO_x species. But research groups have reported different values for concentration of these minor species (McMahon et al., 2002), and have indicated that since these conservation species form a very small portion of the total NO_x species, their physiological significance in preserving NO remains unclear. Hence, using the experi-

mental system designed in this study, we found that NO-RBC interactions were dominated by the consumption reaction, forming nitrate and metHb [HbFe(III)].

Conclusions

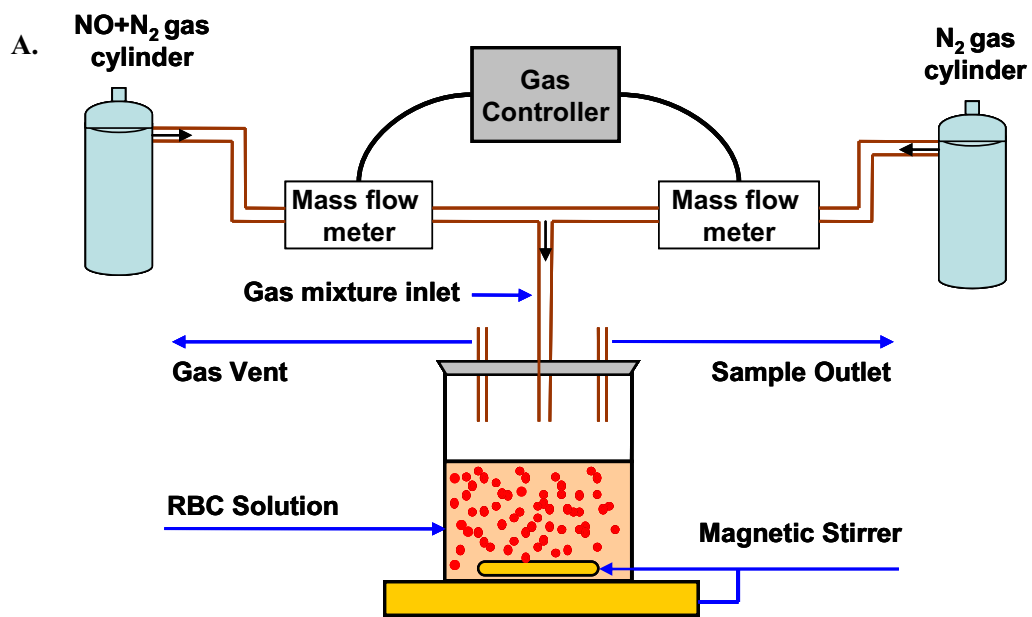
Our results help to explain the mechanism of physiological regulation of NO bioactivity by alteration of RBC concentration and NO delivery rate, and thereby significantly increase our understanding of the interactions of NO with RBCs in the vasculature. The experimental setup allowed maintaining a steady state in the bioreactor as a constant NO flux reacted with the RBC solutions, thus effectively replicating the in-vivo conditions in microcirculation. The setup also could be easily modified to control the NO flux delivered into the solution. Results showed that for oxygenated solution, the nitrite and total NO_x concentrations increased as the hematocrit increased from 5% to 45%. This deeper understanding of the multiple facets of RBC and NO biology will provide important insights into mechanisms of vascular homeostasis and will offer novel therapeutic strategies for treatment of vascular-related pathologies

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B.



Fig. 1. A. The schematic of the experimental system.
B. The experimental system setup in the laboratory.

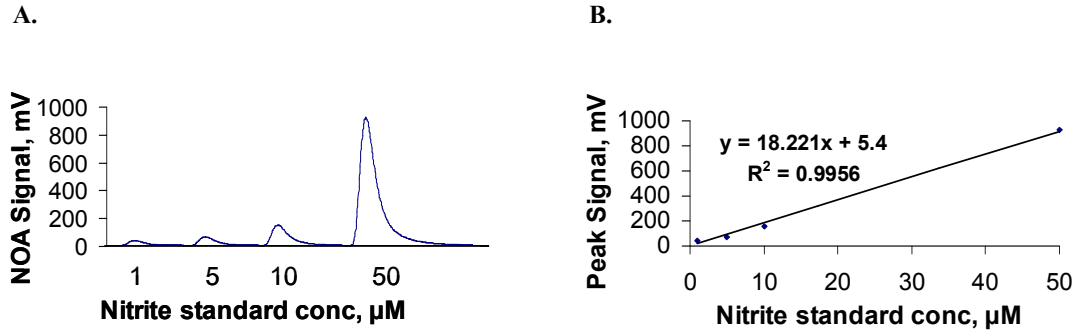


Figure 2: A. Chemiluminescence signal for nitrite standard samples.
 B. Calibration curve for the nitrite standards.

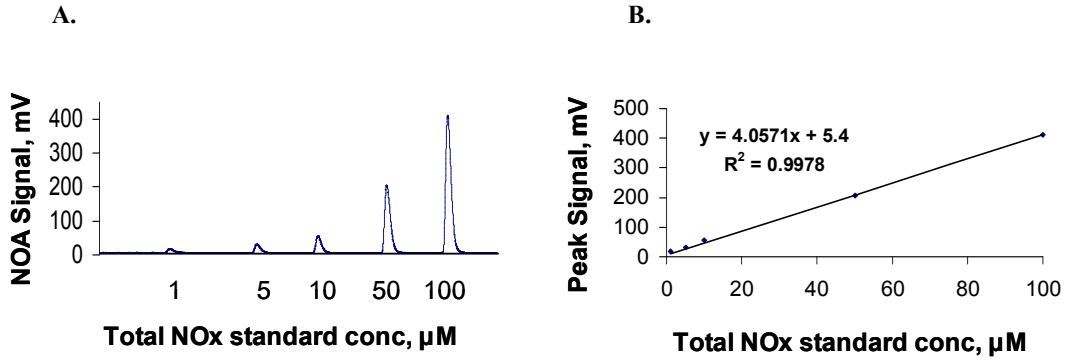


Figure 3: A. Chemiluminescence signal (mV) for total NOx standard samples.
 B. Calibration curve for the total NOx standards.

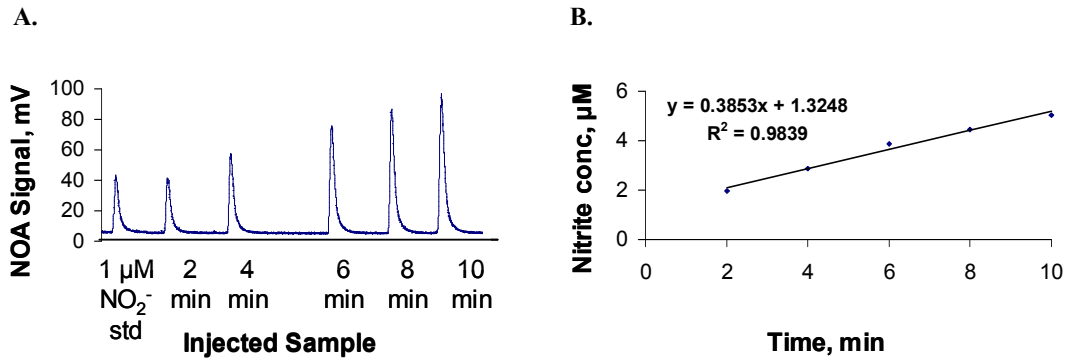


Figure 4: The figures show results of DI water experiment;
 Flow rate: 0.7 ml of 10% NO/ N₂ + 100 ml of 100 % N₂;
 A. Chemiluminescence signal (mV) from NOA;
 B. Nitrite concentration profile for DI water samples taken at regular time (min) intervals.

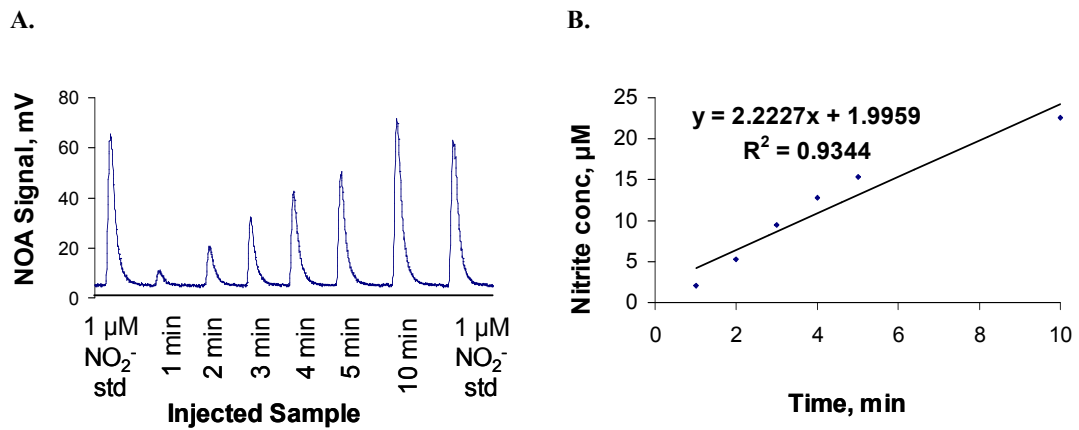


Figure 5: The figures show results of DI water experiment;
 Flow rate: 1.5 ml of 10% NO/ N₂ + 100 ml of 100 % N₂;
 A. Chemiluminescence signal (mV) from NOA;
 B. Nitrite concentration profile for DI water samples taken at regular time (min) intervals.

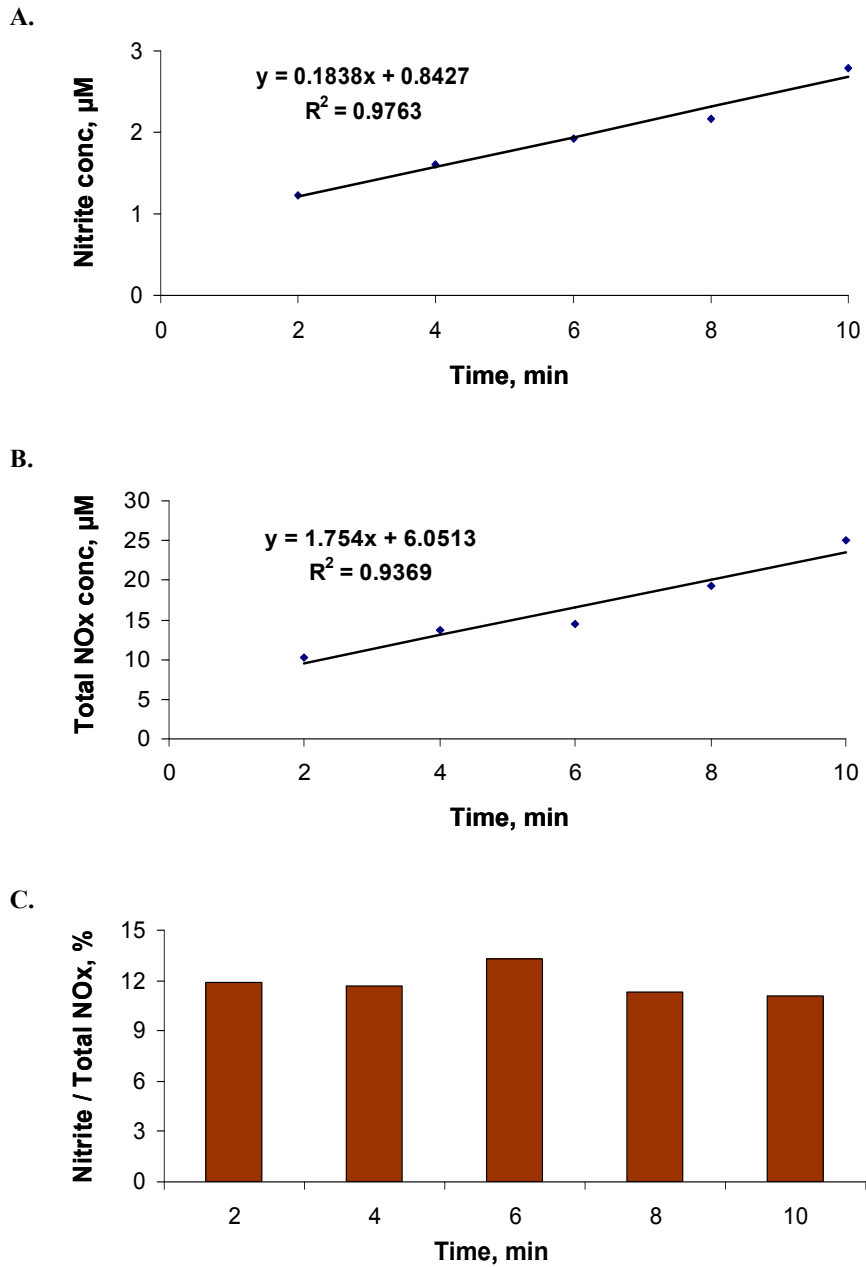


Figure 6: The figures show results of 5% oxy RBC variable time experiment.
 Flow rate: 0.7 ml of 10% NO/ N₂ + 100 ml of 100 % N₂; Total Time: 10 min
 A. Nitrite concentration profile;
 B. Total NOx concentration profile;
 C. Nitrite to total NOx percent ratio for samples taken at regular time (min) intervals.

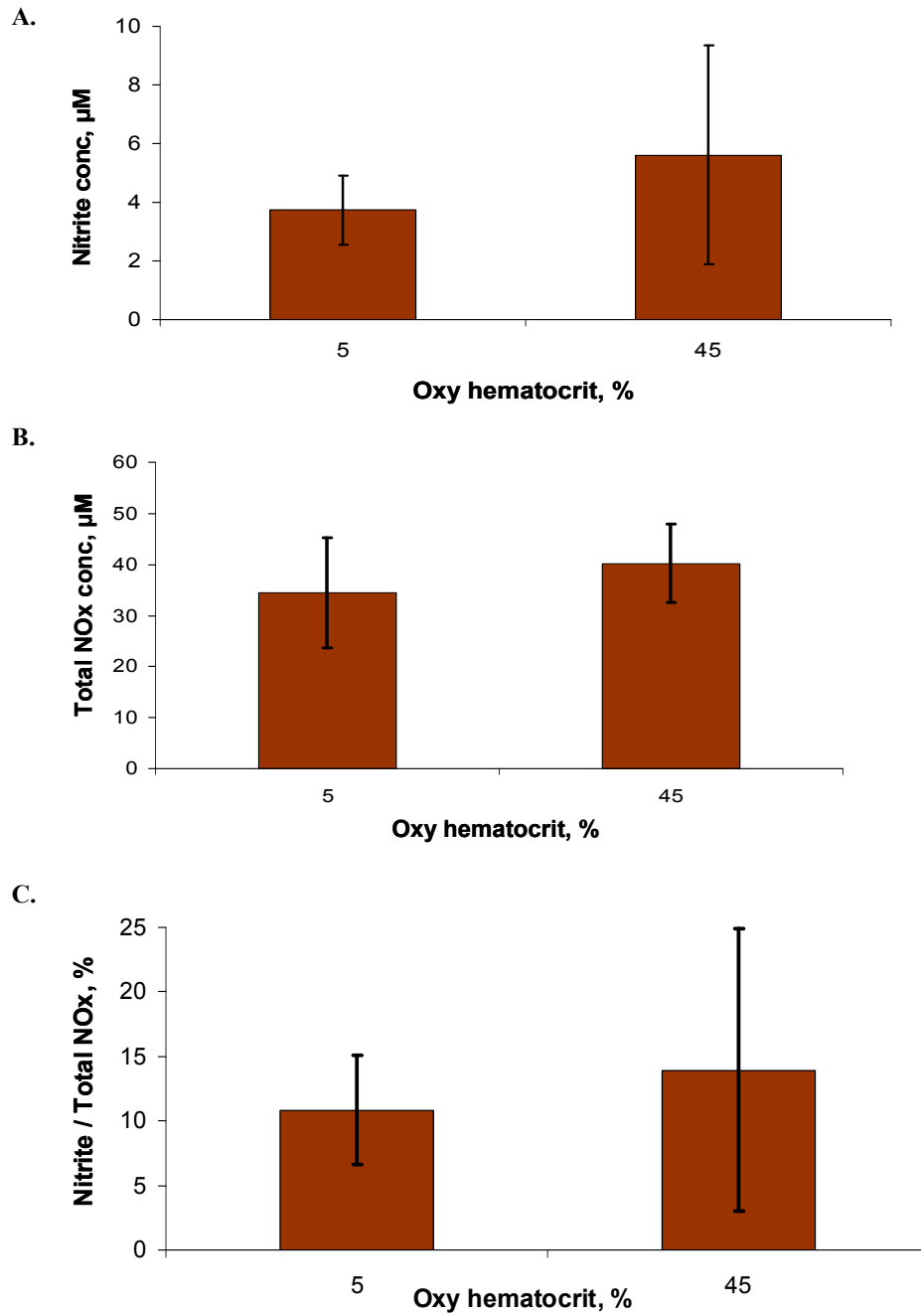


Figure 7: The figures show results of variable Ht experiments in oxygenated conditions
 Flow rate: 0.7 ml of 10% NO/ N₂ + 100 ml of 100 % N₂; Total Time: 10 min
 A. Nitrite concentration in 5 and 45 % Ht oxy solution
 B. Total NOx concentration in 5 and 45% Ht oxy solution
 C. Nitrite to total NOx percent ratio in 5 and 45% Ht oxy solution