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**THE ROLE OF NITRIC OXIDE IN LIVER TRANSPLANTATION: SHOULD IT BE ROUTINELY USED?**

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**ABSTRACT**

Ischemia-reperfusion injury continues to be a major contributor to graft dysfunction, thus supporting the need for therapeutic strategies focused on minimizing organ damage especially with growing numbers of extended criteria grafts being utilized which are more vulnerable to cold and warm ischemia.

Nitric oxide (NO·) is highly reactive gaseous molecule found in air and regarded as a pollutant. Not surprising, it is extremely bioactive, and has been demonstrated to play major roles in vascular homeostasis, neurotransmission, and host defense inflammatory reactions. Under conditions of ischemia, NO·, has consistently been demonstrated to enhance microcirculatory vasorelaxation and mitigate pro-inflammatory responses, making it an excellent strategy for patients undergoing organ transplantation. Clinical studies designed to test this hypothesis have yielded very promising results that includes reduced hepatocellular injury and enhanced graft recovery, without any identifiable complications. How inhaled NO· mediates extra-pulmonary effects remains unclear with the general hypothesis being that it forms a relatively stable, NO· containing intermediate in the circulation, which then mediates systemic effects either directly or after being recycled to NO·. Evidence suggests the NO·-containing intermediates may be plasma S-nitrosothiols (e.g. S-nitrosoalbumin), whereas other studies indicate nitrite anion as a possible mediator. In this article, we discuss the use of inhaled NO· as a way to protect the donor liver graft against ischemia-reperfusion injury in patients undergoing liver transplantation.

**INTRODUCTION**

Liver transplantation has become a viable treatment option for recipients suffering from irreversible liver failure for more than three decades. However, the number of recipients on the waiting list continues to grow due to the major mismatch between organ supply and demand, creating tremendous pressure on for the development of techniques to expand the donor pool. There are ~7.000 liver transplants performed annually with a trend that in increasing due to demand. As a result, according to national transplant registry database from the Organ Procurement and Transplant Network (OPTN), ~1,000 potential recipients on the waiting list die annually. (Figure 1) Therefore, strategies are actively being sought to increase in donor pool. The transplant community has evaluated an option to relax the standard for donors to include donors with suboptimal quality (more damage from anoxic preservation and ischemia-reperfusion injury), including the advanced age donor, prolonged cold and warm ischemia time, and hepatic steatosis. Currently, extended criteria donors make up 5-10% of all donors and this number is increasing. Liver ischemia-reperfusion injury (IRI) remains a major contributor to graft dysfunction, or primary non-function, resulting in increased ICU and hospital stay, increase financial burden, re-transplantation and , in a worst case scenario, death.([1](#_ENREF_1" \o "de Rougemont, 2010 #296))

Nitric oxide (NO·) is an important endogenously produced biological mediator affecting vascular function, metabolic function and host defense mechanisms.([2](#_ENREF_2" \o "Pacher, 2007 #297)) NO· is also an important effector molecule produced by macrophages, dendritic cells critical in host innate and adaptive immunity.([3](#_ENREF_3" \o "Panjwani, 2002 #298), [4](#_ENREF_4" \o "Chen, 2006 #299)) Inhaled NO· has been clinically used to treat pulmonary hypertension due to its vasodilating effect in pulmonary microcirculation without causing any unfavorable systemic hemodynamic changes. More recent evidence has suggested a relative NO· deficiency due to IRI and that the use of preemptive inhaled NO· can attenuate liver IRI during liver transplantation.

**ENDOGENOUS** NO· **AND THE LIVER DURING ISCHEMIA-REPERFUSION**

Injury to the liver due to IRI is a culmination of inflammatory cross talk with the principal participants mentioned previously. ([5](#_ENREF_5" \o "de Rougemont, 2010 #203)) Injury due to ischemia and reperfusion is the main cause of liver injury in response to vascular clamping during hepatic procedures such as hepatectomy and liver transplantation. This insult on the liver results in disturbances of the sinusoidal microcirculation and the generation of a variety of mediators such as reactive oxygen species, cytokines, activation of chemokines and other cell signaling molecules previously mentioned. ([6](#_ENREF_6" \o "Kupiec-Weglinski, 2005 #217))

Hepatic IRI can cause severe hepatocellular injury that contributes to morbidity and mortality both during and after surgery. Reductions of NO· during liver IRI occur and are associated with increased liver injury in both animals and humans ([7](#_ENREF_7" \o "Koken, 1999 #4), [8](#_ENREF_8" \o "Varadarajan, 2004 #259)) In fact, decreased hepatic production of NO· from endothelial nitric oxide synthase or eNOS (responsible for the constitutive production of NO·) within 1 hour of reperfusion in humans undergoing orthotopic liver transplantation was suggested to contribute to the IRI. This event coupled with NO· inactivation due to reactions with abundant reactive oxygen species (ROS), such as O2-•, results in reduced NO· bioavailability. ([9](#_ENREF_9" \o "Ma, 1993 #173), [10](#_ENREF_10" \o "Abe, 2009 #179)) The consequences of this reduced bioavailability include but are not exclusive to increased oxidative stress, increased apoptosis, increased leukocyte adhesion, increased microcirculatory tone, and perturbed mitochondrial function. ([10](#_ENREF_10" \o "Abe, 2009 #179)) Interestingly, restoration with of NO· to more “physiologic” concentrations serves to diminish the liver ischemia injury via countering the adverse actions mentioned previously. Other studies have demonstrated findings that are consistent with the premise that eNOS is crucial for minimizing liver graft injury during liver IRI. For example, liver injury was less in wild type mice compared to eNOS knockouts (eNOS -/-), in addition to the findings that agents given to increase eNOS expression or donate NO· afford greater liver IRI protection. ([11](#_ENREF_11" \o "Duranski, 2006 #9), [12](#_ENREF_12" \o "Katsumi, 2008 #308)) It is also well established that the NO· concentrations during various inflammatory states are significantly increased by increased expression of inducible nitric oxide synthase or iNOS. However, the influence of iNOS and its true contribution in conferring liver protection deserves additional studies. In a rat model of liver IRI, iNOS enzyme activity was significantly increased as per increases in iNOS mRNA expression at 1 and 5 hours after reperfusion, which shows that induction of iNOS has important role in liver ischemia-reperfusion.([13](#_ENREF_13" \o "Hur, 1999 #70)) This is consistent with other studies measuring iNOS expression of conditions of liver IRI. In a porcine model of IRI, intra-portal injection of the selective iNOS inhibitor, aminoguanidine was demonstrated to decrease injury.([14](#_ENREF_14" \o "Isobe, 2000 #73)) In an intriguing study, NOS knockout mice (iNOS -/-) exposed to warm liver IRI demonstrated a much greater magnitude of injury compared to wild type mice. Interestingly, even though injury was greater in the iNOS knockout mice, little to no iNOS mRNA was detectable in the wild type mice. It would appear for now, the true influence of iNOS’s influence on liver injury during ischemia-reperfusion remains unclear.

A number of other endogenous NO·-mediated mechanisms thought to confer protection have been published. For example, NO· has been shown to inhibit caspase proteases via *S*-nitrosylation, thereby inhibiting apoptosis.([15](#_ENREF_15" \o "Maejima, 2005 #89)) This appears to be somewhat concentration dependent. Low physiological concentrations of NO· may inhibit apoptosis. In contrast, higher concentrations may lead to the formation of toxic reactive nitrogen species such as ONOO- or reactive oxygen species which lead to cell necrosis and apoptosis. ([16](#_ENREF_16" \o "Kim, 2001 #109)) Other published mechanisms of NO·-mediated protection include inhibition of nuclear factor kappa B (NF-κΒ) ([17](#_ENREF_17" \o "Marshall, 2004 #131)), reversible inhibition of mitochondrial complex I, and decreased mitochondrial calcium accumulation. ([18](#_ENREF_18" \o "Burwell, 2008 #150)) As to be expected, controversy exists concerning “if” and “how” NO· exerts cellular protection. For instance, in a study by Jaeschke *et al,* ([19](#_ENREF_19" \o "Jaeschke, 1991 #151)), administration of a NO· synthase inhibitor did not attenuate or accentuate liver injury during the initial reperfusion period. Inhibition of NO· was observed not to influence neutrophil migration to the injured sites. While this contradicts a number of other studies, based on their findings, the authors concluded that NO· bioavailability was unlikely to be involved in the post-ischemic oxidant stress and reperfusion injury.([20](#_ENREF_20" \o "Jaeschke, 1992 #170)) Nevertheless, the preponderance of published literature has demonstrated beneficial effects of NO· during liver IRI. These conflicting results might be explained by the fact that the mechanism of NO·-mediated protection varies depending on cell type, cellular compartment concentrations, laboratory methods applied, timing of administration, and duration of NO· exposure.

**THE IMPACT OF EXOGENOUS** NO· **DELIVERY IN ATTENUATING LIVER ISCHEMIA-REPERFUSION INJURY**

Administration of inhaled NO· has demonstrated efficacy both in animal and human studies. ([21-25](#_ENREF_21" \o "Fox-Robichaud, 1998 #284)) NO· inhalation decreases pulmonary and systemic vascular resistance with resultant improvements in tissue oxygenation ￼([26](#_ENREF_26)) increases in renal blood flow, and glomerular filtration rate. ￼([27](#_ENREF_27))([28](#_ENREF_28)) Moreover, inhaled NO· has been demonstrated to exert extra-pulmonary or peripheral effects to the microvasculature as measured by enhanced perfusion and anti-inflammatory properties during post-reperfusion period.￼([29-32](#_ENREF_29)) Due to this seminal work, administration of inhaled NO· has undergone more extensive assessment as an anti-inflammatory therapy in humans subjected to predictable ischemia-reperfusion. ￼([23](#_ENREF_23), [32-34](#_ENREF_32))How inhaled NO· mediates extra-pulmonary effects remains unclear with the general hypothesis being that NO· forms a relatively stable, NO·-containing intermediate in the circulation, which then mediates systemic effects either directly or after being recycled to NO·. ￼([35](#_ENREF_35))Evidence in a feline model of IRI suggested the intermediate may be plasma S-nitrosothiols (e.g. S-nitrosoalbumin), whereas studies in humans and mice indicate nitrite as a possible mediator ￼([35-37](#_ENREF_35)) A key role for nitrite is also indicated by its direct administration protecting against hepatic IRI in murine models and the description of biological mechanisms for nitrite reduction to NO· under ischemic conditions.￼([36](#_ENREF_36), [38](#_ENREF_38)) NO·-containing candidates in the circulation that are relatively labile under biological conditions and may also be formed upon the inhalation of NO· (via nitrosylation or S-nitrosation reactions). These include S-nitrosothiols in the RBC, ferrous-nitrosylhemoglobin (HbNO) and C- or N-nitrosamines (referred to as XNO) ￼([35](#_ENREF_35), [39-41](#_ENREF_39))

Specifically, inhaled NO· (80 ppm, co-administered with 50% oxygen and 50% nitrous oxide, approximately 5 hours) was administered preemptively to healthy patients undergoing lower extremity surgery requiring approximately two hours of tourniquet-induced ischemia and continued until the completion of the surgery. ([34](#_ENREF_34" \o "Mathru, 2007 #265)) Administration of inhaled nitric oxide (80 ppm) significantly attenuated the inflammatory response characterized by reduced expression of CD11b/CD18, P-selectin, and NF-κB compared with the control group. This was accompanied by significant increases in plasma levels of nitrate and nitrite and reduced oxidative stress. In this health cohort inhaled NO· administered at 80 ppm significantly reduced inflammation from IRI in lower extremity. This observation supported the concept that conditions characterized by dysfunction in NO· metabolism, that inhaled NO· may be an effective therapy to replenish systemic NO·, thus mitigating injury. A subsequent randomized controlled clinical trial evaluated the effects of preemptive inhaled NO· in recipients (n=20) undergoing orthotopic liver transplantation.([42](#_ENREF_42" \o "Lang, 2007 #343)) Specifically, inhaled NO· (80 ppm, approximately 4 hours) versus placebo was randomly administered to the recipients just following induction of anesthesia and discontinued at the time of wound closure. Patients who received inhaled NO· significantly demonstrated shorter hospital stay and enhanced recovery of graft function [alanine transaminase (ALT) and aspartate aminotransferase (AST), prothrombin time and activated partial thromboplastin time] by approximately 2-3 days when compared to the placebo group. Intraoperative platelet transfusion was reduced by 50% in recipients who received inhaled NO·. As would be expected plasma nitrite levels were significantly increased in inhaled NO· group compare when compared to placebo. Commonly cited untoward side effects such as the formation of critical levels of methemoglobin, nitrogen dioxide or bleeding complications were not observed. Shortly thereafter, patients at two university centers (Center A and B) were randomized to receive placebo or 80 ppm of inhaled placebo (n = 20/center) or inhaled NO· (80 ppm, n = 20/center) during the operative phase of liver transplantation. MELD scores were significantly higher at Center B (22.5 vs. 19.5, p=0.0001), surgical times were greater at Center B (7.7 vs. 4.5 hrs, p=0.001), and warm ischemia times were greater at Center B (95.4 vs. 69.7 min, p=0.0001). Inhaled NO· enhanced allograft function indexed by liver function tests and reduced hepatobiliary complications at 9-months. Hospital length of stay was not decreased. Inhaled NO· significantly increased concentrations of nitrate, nitrite, and nitrosylhemoglobin, with nitrite being postulated as a protective mechanism. Consistent with previous human studies no adverse metabolic or hematologic effects from were observed between groups. Mean costs of inhaled NO· was $1,020 per transplant. Use of inhaled NO· has also been tested in models of a non-heart beating donor model and steatotic liver, and demonstrated the injury attenuation and enhanced microcirculatory perfusion.([43](#_ENREF_43" \o "Srinivasan, 2012 #380), [44](#_ENREF_44" \o "Nagai, 2013 #262)) These studies are consistent with others demonstrating injury mitigating efficacy when inhaled NO· is utilized prior to predictable IR injury. Replenishing NO· maybe more important in extended criteria donors which appear even susceptible to ischemia (cold and warm)-reperfusion.

Methemoglobinemia is well-documented side effect of high dose supplemental inhaled NO·. Methemoglobin (MetHb) is rapidly formed by oxidation of nitrosylhemoglobin, which is a byproduct from a binding of NO· to hemoglobin in a dose and time dependent manner. MetHb has a fewer hemes to bind oxygen despite that methemoglobin has a higher affinity to oxygen compared to hemoglobin (1,500 times higher affinity compared to carbon monoxide([45](#_ENREF_45" \o "Gibson, 1957 #497))), thus diminishing the oxygen carrying capacity of the blood. MetHb level of 10% of total hemoglobin cause clinically apparent cyanosis, and MetHb level of 35% cause headache, weakness, and dyspnea, and MetHb level of more than 70% are fatal. As aforementioned, clinically significant methemoglobinemia has not been reported when low-dose inhaled NO· is used. Only few case reports of methemoglobinemia has been reported when inhaled NO· was used in high dose (>80ppm). ([46-48](#_ENREF_46" \o "Nakajima, 1997 #498)) However it is worth mentioning that two cases of methemoglobinemia associated with low dose inhaled NO· due to delivery failure have been reported.([49](#_ENREF_49" \o "Taylor, 2001 #501)) In both cases, methemoglobin reductase levels were confirmed to be normal, excluding of heredity methemoglobin reductase deficiency. Authors have speculated that variable (phasic) main flow provided from mechanical ventilator caused periodical accumulation of NO in the inspiratory limb of airway circuit, leading to variable inhaled NO· concentration. Incorporated slow-response chemiluminescent analyzer was unable to detect this fluctuation of inhaled NO·. This fluctuation of NO· was also shown in lung model. *Yamaguchi et al* showed that inhaled NO· was more concentrated when it was instilled more distally in the inspiratory limb of the circuit and it was administered with lower ventilatory flow rates.([50](#_ENREF_50" \o "Yamaguchi, 2000 #502)) They speculated that delivered NO· was diluted by backflow in the NO· tubing from the higher pressure in the circuit in the early inspiratory phase of ventilation. This concentrated NO· in NO· tubing was delivered in the early expiratory phase, leading to fluctuation in NO· concentration. Therefore, inhaled NO· treatment requires caution during administration and other form of supplementation of NO· may be favored in terms of avoiding life-threatening methemoglobinemia.

Other possible complication is the generation of cytotoxic oxidant, “peroxynitrite” by rapidly reacting with superoxide anion.([51](#_ENREF_51)) Peroxynitrite can induce lipid peroxidation and inhibit mitochondrial respiration.([52](#_ENREF_52), [53](#_ENREF_53)) The rate constant for the reaction between NO· and O2 ·- to produce peroxynitrite is 6.7x109 M -1 s -1 and production of peroxynitrite is a diffusion-controlled reaction. Indeed lung damage has been reported after inhaled NO· administration.([54](#_ENREF_54)) Hydrogen gas discover to have an anti-oxidative effect by scavenging peroxynitrite and other hydroxyl radicals.([55](#_ENREF_55)) Hydrogen gas has been shown to ameliorate lipopolysaccharide (LPS)-induced ([56](#_ENREF_56)), ventilator-associated ([57](#_ENREF_57)), and hyperoxia-induced acute lung injury ([58](#_ENREF_58)). Therefore co-administration of hydrogen gas has been investigated to enhance lung protection by NO·.

Again, while the underlying mechanism(s) of how inhaled NO· mitigates injury remains speculative, the possibility of nitrite, an oxidative product of NO· metabolism, playing that protectant role is largely favored. ([36](#_ENREF_36), [37](#_ENREF_37), [59-61](#_ENREF_59)) Consistent with this concept, sodium nitrite has been shown to limit acute IRI in both murine heart and liver of warm ischemia-reperfusion and is associated with decreased myocardial infarct size and hepatocyte apoptosis.([36](#_ENREF_36" \o "Duranski, 2005 #293)) Underlying this protection are biochemical pathways that couple ischemia to nitrite reduction to NO·, which then can mediate cytoprotective effects by multiple possible mechanisms. In a model of murine liver transplantation, harvested syngeneic liver grafts were supplemented with sodium nitrite supplementation either Lactated Ringer’s solution or University of Wisconsin solution during cold ischemia. The syngeneic recipients of liver grafts were also treated with or without nitrite by intraperitoneal injection. Liver enzyme release was significantly reduced in both nitrite-supplemented Lactated Ringer’s solution and University of Wisconsin solution compared to their controls. The protective effect of nitrite was more efficacious with longer cold preservation times. Liver histological examination demonstrated enhanced preservation of morphology and architecture with nitrite treatment. Hepatocellular apoptosis was significantly reduced in the nitrite-treated livers compared their controls. Moreover, liver grafts with extended cold preservation time of 12 - 24 hours demonstrated improved liver tissue histology and function post-reperfusion with either the nitrite-supplemented preservation solution or in nitrite-treated recipients. Interestingly, combined treatment of both the liver graft and recipient did not confer protection. Further clinical studies in the use of inhalation of NO· or injection of NO· donors for extended criteria donor may have a large clinical impact given that there is a surge in use of extended criteria donors to expand donor pool and warrants further investigation

Other potential route of NO· donor administration is per gastrointestinal tract. In fact, dietary intake of nitrate is major source of NO· donor in mammals.([62](#_ENREF_62" \o "Lundberg, 2004 #506)) Dietary nitrate is abundant in many vegetables, and water. Ingested nitrate is absorbed from intestine. One quarter of absorbed nitrate is concentrated in saliva and reduced to nitrite by commensal bacteria.([63](#_ENREF_63" \o "Bryan, 2006 #507)) Inorganic nitrite is converted to NO· in the presence of gastric acid in stomach.([64-66](#_ENREF_64" \o "Benjamin, 1994 #508)) This production pathway of nitric oxide is independent of eNOS (eNOS independent NO production) and accounts for majority of nitrite and nitrate in mammalian body.([65](#_ENREF_65" \o "Rocha, 2012 #509), [66](#_ENREF_66" \o "Gago, 2007 #510)) Absorbed nitrite, nitrate or NO· from small intestine is directly delivered to liver through portal vein. Therefore per oral administration of NO· donor can be a potential route of administration, especially post-transplant period. However supplement of No donor through warrants further investigation towards the clinical use to facilitate recovery of donor liver function.

Other drugs exist, that serve to donate NO· and have been explored as an alternative to the parent compound. ([67-69](#_ENREF_67)) Novel drugs have been developed and used for the delivery of NO· in order to compensate for the very short half-life of NO· *in vivo*. However, there are only two types of NO· donor drugs that are currently used clinically: organic nitrates and sodium nitroprusside. Organic nitrates are the most commonly used NO· donor drugs treatment for coronary artery disease and congestive heart failure because the drugs produce clear clinical responses through their vasodilatory effects. Preparations of drugs include: slow release oral forms, ointments, transdermal patches, nebulizers and traditional intravenous forms. The main limitation of organic nitrates is the induction of drug tolerance with prolonged continuous use. NO· release from nitroglycerin is likely via the enzyme mitochondrial aldehyde dehydrogenase.([70](#_ENREF_70)) On the other hand, the mechanism of NO· release from sodium nitroprusside is more complex as demonstrated by Yang *et al* in a murine model of hepatic IRI. Sodium nitroprusside appears to down-regulate the mRNA expression of several enzymes related to hepatic injury.([12](#_ENREF_12" \o "Katsumi, 2008 #308)) Lastly, enhanced eNOS activation affords hepatoprotection during IRI and serves as yet another potential treatment option. Interestingly, liver preservation solutions supplemented with the agents trimetazidine (TMZ), 5-amino-4-imidazole carboxamide riboside (AICAR) or activated protein C (APC) have demonstrated allograft protection during conditions of cold ischemia.([71](#_ENREF_71)) Below is a summary of some of the novel NO· donor drugs (Table 1).

**Table 1. Nitric Oxide Donors**

|  |  |  |  |
| --- | --- | --- | --- |
| *Model* | *Drugs* | *Outcomes* | *Reference* |
| Canine liver IRI | FK-409,  | - Promote hepatic tissue blood flow, decrease serum Endothelin-1, cytoprotection | (48) (Aiba et al., 2001) |
| Isolated hepatocytes | S-nitroso-N-acetylpenicillamine (SNAP) | - Drug induced the expression of heat shock protein 70 mRNA and protein resulting in cytoprotection from TNFα | (49) (Y. M. Kim et al., 1997) |
| Murine liver IRI | Sodium nitroprusside | - Promote hepatic tissue blood flow after reperfusion - cytoprotection | (50)(Nilsson et al., 2001) |
| Murine liver IRI | PEG-poly SNO-BSA, a sustained release of NO· | - Decreased neutrophils accumulation - Prevented the excessive production of iNOS | (12) (Katsumi et al., 2008) |
| Murine liver IRI | Macromolecule S-nitrosothiols | - Prevented hepatocellular injury | (52)(Katsumi et al., 2009) |

**CONCLUSION**

IRI is a well-defined insult to the liver during periods of interruption and restoration of oxygen delivery, as occurs in certain procedures such as hepatic resections and orthotopic liver transplantation. Relative NO· deficiency seems central in the pathogenesis of this injury. Replacing NO· per either by inhalation, nitrate anion or via NO· donor drugs represents a pragmatic means of mitigating IRI. Clinical studies incorporating inhaled NO· provide solid evidence in mitigating injury from IRI. Inhaled NO· has demonstrated repeated efficacy without any demonstrable metabolic or hematological toxicities. Costs of routine NO· administration during liver transplantation is negligible when the entire spectrum of care is considered. Therefore Nitric oxide has a potential to be a good therapeutic option for organ resuscitation in liver transplantation, especially for the extended criteria (marginal quality) donors, but further investigation is still warranted for routine clinical use.

**FIGURE LEGENDS**

**FIGURE 1. Number of transplants, waiting list additions, and waiting list deaths in the United States between 1995 and 2015.** Number of waiting list additions and deaths are based on the candidates and candidate who is listed more than one place is counted as one candidate. Data are available from <https://optn.transplant.hrsa.gov/data/>.

**FIGURE 2. Delivery of Nitric Oxide to donor liver graft in liver transplantation.** Preemptive treatment with inhaled nitric oxide can attenuate ischemia-reperfusion injury via modulation of a myriad of inflammatory, cellular and vascular mechanisms. *Abbreviations:* NO· *nitric oxide, NO2-, Nitrite, NO3-, nitrate, NOS nitric oxide synthase*

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