Doc Code: PET.SPRE.ACX
Doc Description: Petition for 12-month Accelerated Exam

PTO/SB/28 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U. S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION TO MAKE SPECIAL UNDER ACCELERATED EXAMINATION PROGRAM

Attorney Docket Number	I001-0002USC3	First Named Inventor	James S. Baldassarre
Application Number (if Known)			
Title of Invention	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension		

APPLICANT HEREBY PETITIONS TO MAKE THE ABOVE-IDENTIFIED APPLICATION SPECIAL UNDER THE REVISED ACCELERATED EXAMINATION PROGRAM. See Instruction sheet on page 3.

1. **Claims of the application:**
   a. The application must contain three (3) or fewer independent claims and twenty (20) or fewer total claims. The application may not contain any multiple dependent claims.

   b. **Applicant hereby agrees not to separately argue the patentability of any dependent claim during any appeal** in the application. Specifically, the applicant agrees that the dependent claims will be grouped together with and not argued separately from the independent claim from which they depend in any appeal brief filed in the application (37 CFR 41.37(c)(1)(vii)).

   c. The claims must be directed to **a single invention**.

2. **Interviews:**
   Applicant hereby agrees to have (if requested by examiner):
   a. An interview (including an interview before a first Office action) to discuss the prior art and any potential rejections or objections with the intention of clarifying and possibly resolving all issues with respect to patentability at that time, and

   b. A telephonic interview to make an election without traverse if the Office determines that the claims are not obviously directed to a single invention.

3. **Preexamination Search Statement and Accelerated Examination Support Document:**
   With this petition, applicant is providing: a **preexamination search statement**, in compliance with the requirements set forth in item 8 of the instruction sheet, and an **"accelerated examination support document"** that includes:
   a. An **information disclosure statement** in compliance with 37 CFR 1.98 citing each reference deemed most closely related to the subject matter of each of the claims;

   b. For each reference cited, **an identification of all the limitations of the claims** that are disclosed by the reference specifying where the limitation is disclosed in the cited reference;

   c. A **detailed explanation of how each of the claims are patentable** over the references cited with the particularity required by 37 CFR 1.111(b) and (c);

   d. A concise **statement of the utility** of the invention as defined in each of the independent claims (unless the application is a design application);

   e. An identification of any cited references that may be disqualified as prior art under 35 U.S.C. 103(c) as amended by the CREATE act; and

   f. **A showing of where each limitation of the claims finds support under the first paragraph of 35 U.S.C. 112** in the written description of the specification. If applicable, the showing must also identify: (1) each means- (or step-) plus-function claim element that invokes consideration under 35 U.S.C. 112, ¶6; and (2) the structure, material, or acts that correspond to any means- (or step-) plus-function claim element that invokes consideration under 35 U.S.C. 112, ¶6. If the application claims the benefit of one or more applications under title 35, United St ates Code, the showing must also include where each limitation of the claims finds support under the first paragraph of 35 U.S.C. 112 in each such application in which such support exists.

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PETITION TO MAKE SPECIAL UNDER ACCELERATED EXAMINATION PROGRAM (Continued)

Attorney Docket Number	I001-0002USC3	First Named Inventor	James S. Baldassarre

Attachments:

a.	X	Accelerated Examination Support Document (see item 3 above).
b.		A statement, in compliance with the requirements set forth in item 8 of the instruction sheet, detailing the preexamination search which was conducted.
c.	X	Information Disclosure Statement.
d.	☐	Other (*e.g.*, a statement that the claimed subject matter is directed to environmental quality, energy, or countering terrorism (37 CFR 1.102(c)(2)).

Fees: The following fees must be filed electronically via EFS or EFS-Web:

a.	The basic filing fee, search fee, examination fee, and application size fee (if required) under 37 CFR 1.16.
b.	Petition fee under 37 CFR 1.17(h) - unless the petition is filed with a showing under 37 CFR 1.102(c)(2).

Signature:

Click Remove if you wish to remove this signatory — Remove

Signature		Date	24 June 2010
Name (Print/Typed)	Christopher P. Rogers	Registration Number	36334

Click Add if you wish to add additional signatory — Add

Note: *Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	TBD
Confirmation Number	TBD
Filing Date	Herein
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	TBD
Examiner	TBD
Attorney Docket Number	I001-0002USC3

Pre-Examination Search Document

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This pre-examination search statement is provided in support of the Petition for Accelerated Examination filed herewith.

A pre-examination search was conducted involving U.S. patents and patent application publications, foreign patent documents and non-patent literature as indicated below. The results of the search are provided on an Information Disclosure Statement filed concurrently herewith.

The search primarily includes the following aspects:

- The method of reducing adverse events in patients in need of treating with nitric oxide - excluding patients with pre-existing left ventricular dysfunction.
- The patients have a pulmonary capillary wedge pressure greater than 20mm Hg.
- Patients with left ventricular dysfunction have conditions like systolic or diastolic dysfunction, hypertensive, viral, iodopathic cardiomyopathy, autoimmune disease related cariomyopathy, structural heart disease, idiopathic pulmonary arterial hypertension, pulmonary hypertension cardiomyopathy.

- The patient's population are children and adults.
- Adverse events are pulmonary edema, hypotension, cardiac arrest, ECG changes, hypoxemia, hypoxia and bradycardia.
- The patient in need of nitric oxide inhalation has PCWP<=15mg, PVRI>3micro.sq.meters.
- Left ventricular afterload is minimized by administering a pharmaceutical dosage form comprising nitroglycerin and calcium channel blocker to the patient, using an inter-aortic balloon pump.

8 (A) Pre-examination Search

Details of US Patent Classification Codes used

http://www.uspto.gov/go/classification/

128-Surgery

128/200.14 – Liquid Medicament Atomizer or Sprayer

128/200-24 – Respiratory Method or Device

128/203.15 – Particular treating agent carried by breathes gas

128/203.12 – Means for mixing treating agent with respiratory gas

558- Organic Compounds

558/486 – Glyceryl trinitrate per se (i.e., trinitroglycerin)

423 – Chemistry or Inorganic Compounds

423/405 – Nitric Oxide (NO)

600 – Surgery

600/481 – Cardiovascular

600/513 – Detecting heartbeat electric signal and diverse cardiovascular characteristic

Details of IPC-8 Codes used

http://www.wipo.int/classifications/ipc/ipc8/?lang=en

A61K – Preparations for Medical, Dental, or Toilet Purposes

A61K 33/00 – Medicinal preparations containing inorganic active ingredients

A61K 33/08 – Oxides; Hydroxides

A61P – Specific Therapeutic Activity of Chemical Compounds or Medicinal Preparations

A61P 9/00 – Drugs for disorders of the cardiovascular system

A61P 9/04 – Inotropic agents, i.e. stimulants of cardiac contraction; drugs for heart failure

A61P 9/08 – Vasodilators for multiple indications

A61P 43/00 – Drugs for specific purposes

C01B – Non-Metallic Elements; Compounds Thereof

C01B 21/24 – Nitric oxide (NO)

Dates Conducted: May 10, 2010 and May 17, 2010

Database Searches

Database Service: Legal Advantage

Data Searched: All patents and Non-patent literature

Database Used: MicroPatent, USPTO, European Patent Office/Espacenet, WIPRO, JPO, Google, Springerlink, Wiley Interscience, ScienceDirect, Scirus, Journal of Medicinal Chemistry, ACS Publications, and, Journal of American Academy of Pediatrics.

Search Logic

Search No.	Concept	Keywords
1	Nitric oxide	Nitric oxide, nitrogen monoxide, nitrogen oxide, iNO, NO
2	Inhale	Inhale, breath, gasp
3	Reduce	Reduce, minimize, prevent, avoid, exclude, reject, except, omit
4	Adverse event	Adverse/undesirable/unfavorable/unfavorable event/effect/consequence/indication, side effect, toxicity, toxin
5	Identify	Identify, select, choose, opt, pick, screen, find, segregate, separate, distinguish, take out
6	Left ventricular dysfunction	Left ventricular dysfunction, LVD, diastolic/systolic dysfunction, cardiomyopathy, heart disease
7	Pulmonary Capillary wedge pressure	Pulmonary Capillary wedge pressure, PCWP
8	Respiratory failure	Respiratory failure, Pulmonary edema, hypotension or cardiac arrest, heart failure, heart attack, electrocardiogram/ECG change, hypoxia, hypoxemia, bradycardia

8(B) Search Directed to the Invention

The pre-examination search was directed to the claimed invention, encompassing all the features of the claims and giving the claims their broadest reasonable interpretation.

8(C) Search Directed to the Disclosure

No disclosed features that are unclaimed at this time are currently seen as features that may be claimed later.

8(D) Search Report from a Foreign Patent Office

Search reports from Australia, Japan, and the EPO are attached herewith.

8(E) Statement of Good Faith

All statements above in support of the petition to make special are based on a good faith belief that the search was conducted in compliance with the requirements of this rule.

Respectfully Submitted,

Christopher P. Rogers, Reg. No. 36,334

Date: 21 June 2010

Lee & Hayes, PLLC
601 W. Riverside Avenue, Suite 1400
Spokane, WA 99201

Australian Government
IP Australia

Discovery House, Phillip ACT 2606
PO Box 200, Woden ACT 2606
Australia
Phone: 1300 651 010
International Callers: +61-2 6283 2999
Facsimile: +61-2 6283 7999
Email: assist@ipaustralia.gov.au
Website: www.ipaustralia.gov.au

15 March 2010

RECEIVED 17 MAR 2010

PIZZEYS
Level 14, ANZ Centre
324 Queen Street
Brisbane QLD 4000
Australia

Your Ref: 28686IKA/AMM:Is

Examiner's first report on patent application no. 2009202685
by Ikaria Holdings, Inc.

Last proposed amendment no.

Dear Madam/Sir,

I am replying to the request for normal examination. I have examined the application and I believe that there are lawful grounds of objection to the application. These grounds of objection are:

1. The invention defined in claims 1-30 does not involve an inventive step when compared to the disclosure of each of the following prior art documents*:

   D1: LOH, E. *et al.* "Cardiovascular Effects of Inhaled Nitric Oxide in Patients with Left Ventricular Dysfunction". CIRCULATION, 1994, vol.90: 2780-2785.
   D2: CUJEC, B. *et al.* "Inhaled Nitric Oxide Reduction in Systolic Pulmonary Artery Pressure is Less in Patients with Decreased Left Ventricular Ejection Fraction". CANADIAN JOURNAL OF CARDIOLOGY, 1997, vol.13(9): 816-824.
   D3: ROSALES, A *et al.* "Adverse Hemodynamic Effects Observed with Inhaled Nitric Oxide After Surgical Repair of Total Anomalous Pulmonary Venous Return". PEDIATRIC CARDIOLOGY, 1999, vol.20: 224-226.
   D4: BOCCHI, E. *et al.* "Inhaled Nitric Oxide Leading to Pulmonary Edema in Stable Severe Heart Failure". THE AMERICAN JOURNAL OF CARDIOLOGY, 1994, vol.74: 70-71.
   D5: ARGENZIANO, M. *et al.* "Inhaled Nitric Oxide is not a Myocardial Depressant in a Porcine Model of Heart Failure". THE JOURNAL OF THORACIC AND CARDIOVASCULAR SURGERY, 1998, vol.115: 700-704.

   The problem addressed by the current application is reducing adverse events or serious adverse events associated with inhaled nitric oxide in patients who have pre-existing left ventricular dysfunction.

   The cited art is directed to a problem similar to the applicant's problem, and in searching the problem a person skilled in the art could reasonably be expected to have found, and to have ascertained, understood, and regarded, this prior art as relevant.

   D1 investigated the use of inhalation of the pulmonary vasodilator, nitric oxide (NO), in patients with heart failure due to left ventricular dysfunction (LVD). The cause of heart failure in half the patients was ischemic cardiomyopathy and in the other half it was caused by idiopathic dilated cardiomyopathy (see abstract and Methods: Study Population). Following

administration of NO via a face masks patients showed an increase in the mean pulmonary artery wedge pressure associated with decreases in cardiac index and stroke volume index (see Results). It is suggested that selective pulmonary vasodilation is not desirable in patients with left ventricular failure (see page 2784, last paragraph).

D2 discloses that there have been reports that a decrease in pulmonary vascular resistance following iNO inhalation occurs in patients with LVD as a result of an increase in pulmonary capillary wedge pressure. D2 further investigated the effects of iNO in a group of patients with a broad range of left ventricular function in a randomized manner (see page 817, left col.). Some of the patients received oxygen in addition to NO (see page 818, Study protocol). Three patients with depressed left ventricular ejection fraction (LVEF) presented with pulmonary oedema after administration of nitric oxide (see page 821, left col. 1st paragraph and page 822, right col., lines 4-6). Other adverse events to occur in patiens with depressed LVEF were an increase in pulmonary wedge pressure and decreased pulmonary vascular resistance (the latter patients were also cardiomyopathy patients) (see page 821, right col.). There is a clear suggestion that the use of nitric oxide is limited in patients with pre-existing LVD (see CONCLUSIONS).

D3 discloses a case report of a one month old patient who underwent corrective surgery with pulmonary vein confluence to left atrial anastomosis (see abstract). The patient was treated with NO therapy following development of sudden onset systemic-level pulmonary pressure with concomitant systemic hypotension. However, favourable changes were followed by "rebound" pulmonary hypertension that occurred with concomitant systemic hypotension and central venous pressure. Therapy with NO was discontinued based on the rationale that this episode of pulmonary hypertension may have been caused by left atrial hypertension secondary to a sudden increase in pulmonary blood flow into a noncompliant left atrium and ventricle (see page 225, 4th and 5th paragraphs). As a result, D3 states that NO therapy can be detrimental in patients with LVD and/or cardiomyopathy as these patients may develop pulmonary oedema (see abstract and page 226, left col., last paragraph).

D4 pertains to a study in which patients with refractory heart failure and severe pulmonary hypertension having impaired LVEF and severe and diffuse systolic dysfunction were administered NO via inhalation. Following NO therapy patients presented with an increase in pulmonary wedge pressure and developed pulmonary oedema (see whole document).

D5 discloses that there have been reports of increases in left ventricular end-diastolic pressure and episodes of pulmonary oedema during the clinical use of inhaled nitric oxide (iNO) in patients with pre-existing LVD (see abstract and the introduction).

Each of D1-D5 differs from the instant specification in that they do not specifically disclose excluding patients with LVD from iNO treatment nor the steps of informing a medical provider that excluding patients with LVD from iNO treatment reduces adverse events. However, each of D1-D5 discloses that adverse events occur in patients with pre-existing LVD following administration of iNO and they clearly suggest that precautions should be taken when administering iNO.

Therefore the person skilled in the art would directly and without difficulty, by routine steps, arrive at a solution which is the same as the claimed solution, and therefore the claimed invention lacks an inventive step.

* As found during a national phase search

NOTE: There is a current postponement of acceptance in place. If you overcome all other objections before the expiration of that postponement, the Commissioner will only accept the application at that time if you have filed a clear and unambiguous statement requesting the withdrawal of that postponement. Otherwise, a further adverse report will be issued.

You have 21 months from the date of this report to overcome all my objection(s) otherwise your application will lapse.

You will need to pay a monthly fee for any response you file after 12 months from the date of the first report.

You will also need to pay any annual continuation fees that apply. These will normally be first due five years from the filing date. Please note however that earlier commencement dates apply for divisional applications.

Information about fees may be obtained by phoning 1300 651 010.

Yours faithfully,

EDWINA VANDINE
Patent Examination A
A1 - PBR Plants & Biotechnology
Phone: (02) 6225 6113

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY
Tel. +49 (0)89 2399 - 0
Fax +49 (0)89 2399 - 4465

For any questions about this communication:
Tel.:+31 (0)70 340 45 00

Duckworth, Timothy John
J.A. Kemp & Co.
14 South Square
Gray's Inn
London WC1R 5JJ
GRANDE BRETAGNE

J. A. KEMP & Co
RECD 10 MAY 2010
Action by .....

Date
10.05.10

Reference	Application No./Patent No.
N.108660-TJD	09251949.5 - 2123

Applicant/Proprietor
Ikaria Holdings, Inc.

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 1 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☑ Abstract ☑ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract:

Refund of the search fee

If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Europäisches Patentamt · European Patent Office · Office européen des brevets

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 09 25 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	LOH EVAN ET AL: "Cardiovascular Effects of Inhaled Nitric Oxide in patients With Left Ventricular Dysfunction" CIRCULATION, vol. 90, no. 6, 1994, pages 2780-2785, XP002577161 ISSN: 0009-7322 * the whole document *	1-9	INV. A61K33/00 A61P9/08 A61P9/12
X,D	SEMIGRAN MARC J ET AL: "Hemodynamic effects of inhaled nitric oxide in heart failure" JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, vol. 24, no. 4, 1994, pages 982-988, XP009131903 ISSN: 0735-1097 * the whole document *	1-9	
X,D	HAYWARD C S ET AL: "Inhaled nitric oxide in cardiac failure: Vascular versus ventricular effects" JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, vol. 27, no. 1, 1996, pages 80-85, XP009131904 ISSN: 0160-2446 * the whole document *	1-9	TECHNICAL FIELDS SEARCHED (IPC) A61K
X	OVODOV ET AL: "Nitric oxide: Clinical applications" SEMINARS IN ANESTHESIA, SAUNDERS, CO, NEW YORK, NY, US LNKD- DOI:10.1053/SA.2000.6785, vol. 19, no. 2, 1 June 2000 (2000-06-01), pages 88-97, XP005426335 ISSN: 0277-0326 * page 90, column 1 * * page 93, column 2 - page 94 *	1-9	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	13 April 2010	Albrecht, Silke

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.02 (P04C01)

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 09 25 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	HENRICHSEN ET AL: "Inhaled nitric oxide can cause severe systemic hypotension" JOURNAL OF PEDIATRICS, MOSBY-YEAR BOOK, ST. LOUIS, MO, US LNKD-DOI:10.1016/S0022-3476(96)70230-5, vol. 129, no. 1, 1 July 1996 (1996-07-01), page 183, XP022199226 ISSN: 0022-3476 * the whole document *	1-9	
X	ADATIA ET AL: "Inhaled nitric oxide and hemodynamic evaluation of patients with pulmonary hypertension before transplantation" JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, ELSEVIER, NEW YORK, NY, US LNKD- DOI:10.1016/0735-1097(95)00048-9, vol. 25, no. 7, 1 June 1995 (1995-06-01), pages 1656-1664, XP005857183 ISSN: 0735-1097 * page 1663, column 1 *	1-9	TECHNICAL FIELDS SEARCHED (IPC)
X	CUJEC BIBIANA ET AL: "Inhaled nitric oxide reduction in systolic pulmonary artery pressure is less in patients with decreased left ventricular ejection fraction" CANADIAN JOURNAL OF CARDIOLOGY, vol. 13, no. 9, 1997, pages 816-824, XP002577162 ISSN: 0828-282X * the whole document *	1-9	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	13 April 2010	Albrecht, Silke

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A : technological background
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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 09 25 1949

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	FINDLAY G P: "Paradoxical haemodynamic response to inhaled nitric oxide" INTERNATIONAL JOURNAL OF INTENSIVE CARE 1998 GB, vol. 5, no. 4, 1998, pages 134-139, XP001536771 ISSN: 1350-2794 * the whole document *	1-9	
X,D	BOCCHI E A ET AL: "Inhaled nitric oxide leading to pulmonary edema in stable severe heart failure" AMERICAN JOURNAL OF CARDIOLOGY, CAHNERS PUBLISHING CO., NEWTON, MA, US LNKD-DOI:10.1016/0002-9149(94)90496-0, vol. 74, no. 1, 1 July 1994 (1994-07-01), pages 70-72, XP023278686 ISSN: 0002-9149 [retrieved on 1994-07-01] * the whole document *	1-9	

TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	13 April 2010	Albrecht, Silke

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

(Translation of Official Action)

NOTIFICATION OF REASON FOR REJECTION

Mailed: February 23, 2010

Japanese Patent Application No. 2009-157623

Applicant: IKARIA HOLDINGS, INC.

The present application should be rejected for the following reason(s). If the applicant has any argument against the reason(s), an Argument must be filed within three months of the mailing date of this Official Action.

REASON 1
The present invention as claimed in the following claim(s) is unpatentable under Article 29, paragraph 1, sub-paragraph 3 of the Japanese Patent Law as being anticipated by the following publication(s) distributed in Japan or elsewhere or as being identical with an invention made available to the public through electric telecommunications prior to the filing of the present application.

REASON 2
The present invention as claimed in the following claim(s) is unpatentable under Article 29, paragraph 2 of the Japanese Patent Law since the invention could have been easily made by those skilled in the art to which it pertains on the basis of the invention(s) described in the following publication(s) distributed in Japan or elsewhere or an invention/inventions made available to the public through electric telecommunications prior to the filing of the present application.

NOTE:

Citation 1: Inglessis, I. *et al.*, Journal of the American College of Cardiology, 2004, Vol. 44, No. 4, pp. 793-798

Citation 2: Loh, E. *et al.*, Circulation, 1994, 90, pp. 2780-2785

Citation 3: Steinhorn, R.H. *et al.*, Pulmonary Hypertension, Persistent-Newborn, emedicine, updated Apr. 19, 2007
[http://emedicine.medscape.com/article/898437-overview]

Citation 4: BOCCHI, E.A. *et al.*, The American Journal of Cardiology, 1994, Vol. 74, pp. 70-72

A.

Reasons 1 and 2/ Claims 1 to 14/ Citation 1

Citation 1 discloses that inhaled nitric oxide is known as a selective pulmonary vasodilator (Abstract), and that inhaled nitric oxide, when administered to patients with right ventricular myocardial infarction and cardiogenic shock, reduced the pulmonary arterial pressure (Abstract). Citation 1 also discloses that the inhalation of nitric oxide is known to decrease pulmonary vascular tone in adults and children with pulmonary hypertension (page 793, right column, lines 11 to 6 from the bottom), and that nitric oxide is delivered by means of a ventilator or is mixed with oxygen (page 795, left column, "NO administration"). Especially, Table 2 presents hemodynamic parameters of target patients at the time of study enrollment, indicating that most of the patients have a pulmonary capillary wedge pressure (PCWP) of less than 20 mmHg.

In light of the present specification (paragraph [0013]), the patients of Citation 1 having a PCWP of less than 20 mmHg are not deemed to have pre-existing left ventricular dysfunction (LVD).

Thus, the present invention as claimed in claims 1 to 14 is indistinguishable from the invention disclosed in Citation 1.

(The present invention and the invention disclosed in Citation 1 are identical in active ingredient and target patients, and thus are deemed to necessarily provide the same functions/effects.)

B.

Reason 2/ Claims 1 to 14/ Citations 1 to 4

Inhaled nitric oxide is well known as a selective pulmonary vasodilator, as disclosed in Citation 1.

On the other hand, Citation 2 (for example, Abstract) discloses that inhaled nitric oxide, when administered to patients with left ventricular dysfunction, may cause a decrease in pulmonary vascular resistance associated with an increase in left ventricular filling pressure, leading to the risk of the occurrence of adverse events.

Citation 3 (for example, see Abstract and "Treatment with iNO") discloses that, although inhaled nitric oxide is used for the treatment of pulmonary hypertension of newborns, patients suffering from congenital cardiac disease characterized by left

ventricular outflow tract obstruction and severe left ventricular dysfunction have a contraindication to the treatment with inhaled nitric oxide.

Citation 4 (page 71, left column, lines 13 to 15) discloses that inhaled nitric oxide, when administered to patients with severe heart disease, may cause pulmonary edema.

In view of the above, it would have been obvious to those skilled in the art to exclude patients with pre-existing left ventricular dysfunction from patients to be treated with a selective pulmonary vasodilator, in order to avoid the occurrence of adverse events, based on Citations 1 to 4.

Further, the present invention as claimed in claims 1 to 14 is not deemed to provide particularly remarkable advantages, in view of Citations 1 to 4.

REASON 3
The present application should be rejected on the grounds that the recitation of the claim(s) fails to meet the requirement of Article 36, paragraph 6, sub-paragraph 2 of the Japanese Patent Law in the following respect(s).

NOTES:

(1) The abbreviations "PAPm," "PCWP" and "PVRI" are unclear in meaning.

(2) The term "near" renders the scope of the claimed invention unclear, and thus is inappropriate as an expression for use in the claims.

Background Art Information*

Field of Search: IPC A61K33/00

*The information provided herein constitutes no reason for rejection.

拒絶理由通知書

特許出願の番号	特願２００９－１５７６２３
起案日	平成２２年　２月　９日
特許庁審査官	辰己　雅夫　４４９８　４Ｃ００
特許出願人代理人	吉武　賢次（外　３名）　様
適用条文	第２９条第１項、第２９条第２項、第３６条

この出願は、次の理由によって拒絶をすべきものです。これについて意見がありましたら、この通知書の発送の日から３か月以内に意見書を提出してください。

理　由

１．この出願の下記の請求項に係る発明は、その出願前に日本国内又は外国において、頒布された下記の刊行物に記載された発明又は電気通信回線を通じて公衆に利用可能となった発明であるから、特許法第２９条第１項第３号に該当し、特許を受けることができない。

２．この出願の下記の請求項に係る発明は、その出願前に日本国内又は外国において頒布された下記の刊行物に記載された発明又は電気通信回線を通じて公衆に利用可能となった発明に基いて、その出願前にその発明の属する技術の分野における通常の知識を有する者が容易に発明をすることができたものであるから、特許法第２９条第２項の規定により特許を受けることができない。

３．この出願は、特許請求の範囲の記載が下記の点で、特許法第３６条第６項第２号に規定する要件を満たしていない。

記　　（引用文献等については引用文献等一覧参照）

A.
・理由　１，２
・請求項　１－１４
・引用文献等　１
・備考：
引用文献１には、吸入用一酸化窒素は選択的肺血管拡張剤として知られていること（Abstract）、右心室心筋梗塞および心臓ショックを有する患者に吸入用一

酸化窒素を投与したところ、肺動脈圧が減少したこと（Abstract）が記載されている。同文献にはまた、一酸化窒素の吸入は、成人や小児の肺高血圧患者の肺血管緊張を減少させることが知られていること（p.793 右欄下から11行-下から6行）、ベンチレーターを使用して送達することや酸素と混合すること（p.795 左欄"NO administration"）についても記載されており、特に、Table2には、対照患者の試験登録時の血行動態パラメーターが記載され、多くの患者の肺毛細血管楔入圧（ＰＣＷＰ）が２０ｍｍＨｇ未満であることが示されている。

ここで、本願明細書【００１３】の記載からみて、引用文献１のＰＣＷＰが２０ｍｍＨｇ未満の患者は、先在性左心室機能障害（ＬＶＤ）を有していないものと認められる。

してみると、請求項１－１４に係る発明は引用文献１に記載された発明と区別することができない。

（本願発明と引用文献１記載の発明は、有効成分と対象患者が同一であるから、当然に同様の作用効果を奏するものといえる。）

Ｂ.

・理由　２

・請求項　１－１４

・引用文献等　１－４

上記の引用文献１に記載されるように、吸入用一酸化窒素は選択的肺血管拡張剤として周知のものである。

一方、引用文献２（Abstract等）には、左心室機能不全の患者に吸入用一酸化窒素を投与すると、左心充満圧の上昇に伴う肺血管抵抗の低下を引き起こし、有害事象が生ずる可能性があることが記載されている。

引用文献３（Abstract, "Treatment with iNO"等）には、新生児肺高血圧の治療に吸入用一酸化窒素が用いられるものの、左心室流路障害で特徴づけられる先天性心疾患や、重篤な左心室機能不全の患者に対しては、吸入用一酸化窒素による治療は禁忌であると記載されている。

引用文献４（p.71 左欄第13-15行）には、重篤な心疾患の患者に吸入用一酸化窒素を投与すると、肺水腫を引き起こす可能性があることが記載されている。

してみると、引用文献１－４の記載に基づき、有害事象の発生を避けるべく、選択的肺血管拡張剤の対象患者から、先在性左心室機能障害を有する患者を除外することは当業者が容易に想到し得たことである。

そして、請求項１－１４に係る発明が引用文献１－４の記載からみて格別顕著な効果を奏するとも認められない。

Ｂ.

・理由　３

（１）

・請求項　7

「ＰＡＰｍ」、「ＰＣＷＰ」、「ＰＶＲＩ」は略語であり、その意味が不明である。

（２）

・請求項　１０

「ほぼ」なる記載は発明の範囲を不明確とするものであって、特許請求の範囲の記載として適切でない。

引　用　文　献　等　一　覧

1.Inglessis, I. et al., Journal of the American College of Cardiology, ２００４年, Vol.44, No.4, p.793-798

2.Loh, E. et al., Circulation, １９９４年, 90, p.2780-2785

3.Steinhorn, R.H. et al., Pulmonary Hypertension, Persistent-Newborn, e medicine, Updated Apr 19, 2007 [http://emedicine.medscape.com/article/898437-overview]

4.BOCCHI, E.A. et al., The American Journal of Cardiology, １９９４年, Vol.74, p.70-72

（注）法律又は契約等の制限により、提示した非特許文献の一部又は全てが送付されない場合があります。

先行技術文献調査結果の記録

・調査した分野　　ＩＰＣ　Ａ６１Ｋ３３／００

この拒絶理由通知の内容に関するお問い合わせ、または面接のご希望がございましたら下記までご連絡下さい。

特許審査第三部医療　高橋樹理
ＴＥＬ．０３（３５８１）１１０１　内線３４５０
ＦＡＸ．０３（３５８１）１３４２

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	TBD
Confirmation Number	1376
Filing Date	Herein
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	1614
Examiner	TBD
Attorney Docket Number	I001-0002USC3

ACCELERATED EXAMINATION SUPPORT DOCUMENT

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

This Accelerated Examination Support Document (AESD) is submitted in support of the Petition for Accelerated Examination filed herewith.

Claims 1-20 are currently pending in the continuation application. A listing of the claims starts on page 2 herein.

The remaining sections of the AESD begin on page 6. Consideration and grant of the Petition to Accelerate Examination is respectfully requested.

CLAIMS

1. A method of reducing the risk or preventing the occurrence, in a patient being a neonate or near-term neonate, of one or more adverse events or serious adverse events associated with a medical treatment comprising inhalation of nitric oxide, said method comprising:

a. providing pharmaceutically acceptable nitric oxide gas to a medical provider; and,

b. informing the medical provider that excluding said patients who have pre-existing left ventricular dysfunction from said treatment reduces the risk or prevents the occurrence of the adverse event or serious adverse event associated with said medical treatment.

2. The method of claim 1, wherein the adverse event or serious adverse event is one or more of pulmonary edema, hypotension, cardiac arrest, electrocardiogram changes, hypoxemia, hypoxia and bradycardia, or, associations thereof.

3. The method of claim 1, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema in the patient.

4. The method of claim 3, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker to the patient.

5. The method of claim 3, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

6. A method of reducing the risk or preventing the occurrence, in a patient being a neonate or near-term neonate, of one or more adverse events or serious adverse events associated with a medical treatment comprising inhalation of nitric oxide, said method comprising:

a. providing pharmaceutically acceptable nitric oxide gas to a medical provider; and,

b. informing the medical provider that such patients that have pre-existing left ventricular dysfunction experience an increased rate of adverse events or serious adverse events associated with said medical treatment.

7. The method of claim 6, further comprising informing the medical provider of a risk of an adverse event or a serious adverse event in such patients who have a pulmonary capillary wedge pressure greater than 20 mm Hg.

8. The method of claim 6, further comprising informing the medical provider that there is a risk associated with using inhaled nitric oxides in such patients who have pre-existing or clinically significant left ventricular dysfunction and that such risk should be evaluated on a case by case basis.

9. The method of claim 6, further comprising informing the medical provider that there is a risk associated with using inhaled nitric oxide in such patients who have left ventricular dysfunction.

10. The method of claim 6, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema in the patient.

11. The method of claim 10, wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker to the patient.

12. The method of claim 10, wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

13. A method of reducing one or more adverse events or serious adverse events in an intended patient population comprising neonates or near-term neonates in need of being treated with inhaled nitric oxide comprising:

a. identifying a patient eligible for inhaled nitric oxide treatment;

b. evaluating and screening the patient to identify if the patient has pre-existing left ventricular dysfunction; and

c. excluding from inhaled nitric oxide treatment any patient having pre-existing left ventricular dysfunction.

14. The method of claim 13, wherein the patient having pre-existing left ventricular dysfunction also exhibits a pulmonary capillary wedge pressure greater than 20 mm Hg.

15. The method of claim 13, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema in the patient.

16. The method of claim 15,

wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker to the patient, or,

wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

17. A method of reducing the risk or preventing the occurrence, in a patient being a neonate or near-term neonate, of one or more adverse events or serious adverse events associated with a medical treatment comprising inhalation of nitric oxide, the method comprising:

a. identifying said patient in need of receiving inhalation of nitric oxide treatment;

b. evaluating and screening the patient to identify if the patient has pre-existing left ventricular dysfunction; and

c. administering the inhalation of nitric oxide if the patient has not been diagnosed as having pre-existing left ventricular dysfunction, thereby reducing the risk or preventing the occurrence of the adverse event or significant adverse event associated with the inhalation of nitric oxide treatment.

18. The method of claim 17, wherein the patient diagnosed as having pre-existing left ventricular dysfunction also exhibits a pulmonary capillary wedge pressure greater than 20 mm Hg.

19. The method of claim 17, further comprising reducing left ventricular afterload to minimize or reduce the risk of the occurrence of an adverse event or serious adverse event being pulmonary edema in the patient.

20. The method of claim 19,

wherein the left ventricular afterload is minimized or reduced by administering a pharmaceutical dosage form comprising nitroglycerin or calcium channel blocker to the patient, or,

wherein the left ventricular afterload is minimized or reduced using an intra-aortic balloon pump.

9(A) References Deemed Most Closely Related

An Information Disclosure Statement in compliance with 37 CFR 1.98 has been filed herewith citing each of the following references deemed most closely related to the subject matter of the claims. The references listed in the IDS submitted herewith but not listed in this Petition are not closely related to the claimed invention particularly as compared to the references listed and discussed herein.

List of Most Closely Related References

Use of Nitric Oxide, American Academy of Pediatrics, Pediatrics, Vol. 106, No. 2, August 2000, pp. 344-345. ("AAP").

Lipshultz, SE, Ventricular dysfunction clinical research in infants, children and adolescents, Progress in Pediatric Cardiology, 12 (2000):1-28. ("Lipshultz").

The Neonatal Inhaled Nitric Oxide Study Group, Inhaled Nitric Oxide In Full-Term and Nearly Full-Term Infants With Hypoxic Respiratory Failure, N Engl J Med, 1997, Vol. 336, No. 9, pp. 597-604. Correction at N Engl J Med 1997;337:434. ("NINOS").

Hayward CS et al., Inhaled Nitric Oxide in Cardiac Failure: Vascular Versus Ventricular Effects, J Cardiovasc Pharmacol, Vol. 27, No. 1, 1996. ("Hayward 1996").

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Loh, E., et al., Cardiovascular Effects of Inhaled Nitric Oxide in Patients with Left Ventricular Function, Circulation, 1994, Vol. 90:2780-2785. ("Loh").

Inglessis I et al., Hemodynamic effects of inhaled nitric oxide in right ventricular myocardial infarction and cardiogenic shock, JACC, Vol. 44, No. 4, August 18, 2004:793-8. ("Inglessis 2004").

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Bocchi EA et al., Inhaled Nitric Oxide Leading to Pulmonary Edema in Stable Severe Heart Failure, The American Journal of Cardiology, Vol. 74, July 1, 1994. ("Bocchi").

Cujec, B., et al., Inhaled Nitric Oxide Reduction in Systolic Pulmonary Artery Pressure is Less in Patients with Decreased Left Ventricular Ejection Fraction, Canadian Journal of Cardiology, 1997, vol. 13(9):816-824. ("Cujec").

Rosales, A, et al., Adverse Hemodynamic Effects Observed with Inhaled Nitric Oxide After Surgical Repair of Total Anomalous Pulmonary Venous Return, Pediatric Cardiology, 1999, vol. 20:224-226. ("Rosales").

Argenziano, M, et al., Inhaled Nitric Oxide is not a Myocardial Depressant in a Porcine Model of Heart Failure, The Journal of Thoracic and Cardiovascular Surgery, 1998, vol. 115:700-704. ("Argenziano").

Steinhorn RH et al., Inhaled nitric oxide enhances oxygenation but not survival in infants with alveolar capillary dysplasia, J Pediatr, March 1997;130(3):417-22 (3rd). ("Steinhorn 1997").

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Krasuski RA et al., Inhaled Nitric Oxide Selectivity Dilates Pulmonary Vasculature in Adult Patients With Pulmonary Hypertension, Irrespective of Etiology, JACC, Vol. 36, No. 7, December 2000:2204-11. ("Krasuski").

Semigran MJ et al., Hemodynamic Effects of Inhaled Nitric Oxide in Heart Failure, JACC, Vol. 24, No. 4, October 1994:982-8. ("Semigran").

Dickstein ML et al., A Theoretic Analysis of the Effect of Pulmonary Vasodilation on Pulmonary Venous Pressure: Implications for Inhaled Nitric Oxide Therapy, J Heart Lung Transplant, 1996;15:715-21. ("Dickstein").

Henrichsen T et al., Inhaled nitric oxide can cause severe systemic hypotension, The Journal of Pediatrics, Vol. 129, No. 1, p. 183, 1July1996. ("Henrichsen").

Ovodov KJ et al., Nitric Oxide: Clinical Applications, Seminars in Anesthesia, Perioperative Medicine and Pain, Vol. 19, No. 2, June 2000, pp. 88-97. ("Ovodov")

Adatia I et al., Inhaled Nitric Oxide and Hemodynamic Evaluation of Patients With Pulmonary Hypertension Before Transplantation, JADD, Vol. 25, No. 7, June 1995, pp. 1656-64. ("Adatia").

Findlay GP et al., Paradoxical haemodynamic response to inhaled nitric oxide, International Journal of Intensive Care, Vol. 5, No. 4, 1998, pp. 134-139. ("Findlay").

9(B) Identification of Limitations Disclosed by References

AAP:

In August 2000, the Committee on Fetus and Newborn of the American Academy of Pediatrics issued a report on the use of iNO in infants. A relevant portion states:

> iNO should be administered using FDA-approved devices that are capable of administering iNO in constant concentration ranges in parts per million or less throughout the respiratory cycle. Infants who receive iNO therapy should be monitored according to institutionally derived protocols designed to avoid the potential toxic effects associated with iNO administration. These effects include methemoglobinemia (secondary to excess nitric oxide concentrations), direct pulmonary injury (attributable to excess levels of nitrogen dioxide), and ambient air contamination.

(P. 344, 2nd col.). AAP also lists seven RECOMMENDATIONS. (Pp. 344-345). However, AAP is completely silent respecting excluding from iNO treatment any child patient diagnosed with pre-existing left ventricular dysfunction.

Lipshultz:

Lipshultz teaches that data or information gleaned from iNO studies in adults does not correlate or is otherwise probative of iNO studies in children. In other words, children with ventricular dysfunction must be diagnosed, understood, and treated differently than adult patients diagnosed with ventricular dysfunction. Relevant statements are found in the abstract:

> Many changing developmental properties of the pediatric myocardium and differences in the etiologies of ventricular dysfunction in children compared with adults [exist] ... invalidating the concept that children can safely be considered small adults for the purpose of understanding heart failure pathophysiology and treatment.

At page 2, the author states:

> The disease processes resulting in ventricular dysfunction are often different in children than adults. Many pediatric conditions have no close analogies in the adult ... [hence] the effects of intervention may be unlike those seen in adults.

And, at page 5, the author states:

> when trying to understand the proper therapy for children with ventricular dysfunction it is usually important not to view the child as a small adult and extrapolate the effects of ventricular dysfunction therapy for adult ischemia or post-infarction patients to the child where a multitude of non-ischemic, non post-infarction etiologies exist.

NINOS:

At page 597 under "Conclusions" it states:

> Nitric oxide therapy reduced the use of extracorporeal membrane oxygenation, but had no apparent effect of mortality, in critically ill infants with hypoxic respiratory failure.

As set forth in the "Results" section on page 597, the study included 121 infants in the control group and 114 infants in the nitric oxide group. Left ventricular dysfunction was not mentioned.

As to patient eligibility, NINOS states:

> Infants born at 34 or more weeks of gestation who required assisted ventilation for hypoxic respiratory failure and had an oxygenation index of at

> least 25 on two measurements made at least 15 minutes apart were eligible for the trial.
> Infants were considered ineligible for the study if they were more than 14 days old, had a congenital heart disease, or if it had been decided not to provide full treatment.

(P. 598 under "Study Patients").

Hayward 1996:

The ten patients (19 to 59 years old) in this study had severe LV dysfunction and secondary pulmonary hypertension. (See p. 81 under "Methods" and Results" headings). iNO was administered in 10, 20 and 40 ppm doses. (Id. at 2nd col.). The study concludes stating:

> Our results confirm the safety and utility of INO in short-term assessment of pulmonary hypertension in patients with severe cardiac impairment. The possibility of worsening cardiac function in some patients is worrisome, however, and suggests that INO should be used cautiously in such patients and only in combination with other treatments that have been shown to improve LV function. Safety guidelines for the use of INO were recently formulated. We recommend that these guidelines be expanded to include caution regarding the use of INO in patients with severe LV dysfunction. Further study of the haemodynamic effects of INO on the left ventricle is needed.

(P. 84).

Hayward 1997:

This study was conducted in eleven adults being 51-69 years old with normal LV function. (P. 49, under "Methods" heading). The objective of the study was to determine the effects of iNO on load-independent indexes of normal human LV function. (Id. under "Objectives" heading). The results were that iNO had no effect on steady state LV pressure, volume, contractility duration, active relaxation, diastolic compliance or PVR. (Id. under "Results" heading). Thus, it was concluded that 20 ppm of iNO does not significantly affect normal LV function. (Id. under "Conclusions" heading).

Roberts:

The study included 30 newborn infants having "severe hypoxemia even though they were receiving mechanical ventilation at an FiO_2 of 1.0" (p. 606 under "Criteria for Eligibility") to determine whether iNO decreases severe hypoxemia in infants with persistent pulmonary hypertension. (See Abstract and Results, p. 605). The study concluded that "[i]nhaled nitric oxide improves systemic oxygenation in infants with persistent pulmonary hypertension and may reduce the need for more invasive treatments." (See Conclusions, p. 605).

Roberts further states under the "Criteria for Eligibility" heading:

> Infants were excluded from the study if they had any of the following: previous treatment with extracorporeal membrane oxygenation or high-frequency oscillatory or jet ventilation, a congenital diaphragmatic hernia or suspected lung hypolasia, structural cardiac lesions (other than a patent ductus arteriosus), uncorrected hypotension (a mean aortic pressure below 40 mm Hg) or polycythemia (an arterial hematocrit of at least 70 percent), an unevacuated pneumothorax, or a phenotype consistent with a lethal chromosomal abnormality. Since infants who have received exogenous surfactant without sustain increases in systemic oxygenation have responses to inhaled nitric oxide similar to those of infants not previously treated with surfactant, they were not excluded from the study.

Loh:

This is a study of 19 patients with an average age of 52 +/- 3 years. (See p. 2780 under "Study Population" heading). These adult patients suffered from ischemic cardiomyapathy (heart failure due to coronary artery disease and resultant partial cardiac muscle death) and idiopathic dilated cardiomyopathy. (Id.). Fourteen of the patients were diagnosed with left ventricular dysfunction. (See p. 2780 under "Methods and Results" heading).

Loh discloses:

> The most prominent hemodynamic effect of NO inhalation was the increase in pulmonary artery wedge pressure (median increase 26%).
> Thus, more severe LV dysfunction (as evidenced by higher left heart filling pressures, lower stroke volume, and larger LV cavity size) was present in the

patients who had the largest increases in pulmonary artery wedge pressure with inhaled NO.

(P. 2782 under "Hemodynamic Determinants of an Increase in Pulmonary Artery Wedge Pressure With Inhaled NO" heading).

Loh further discloses:

The major finding of this study is that in patients with reactive pulmonary arterial hypertension secondary to LV failure, inhalation of NO causes reciprocal changes in the PVR (decrease) and LV filling pressure (increase). In contrast, in patients with LV failure, we found that inhalation of NO is associated not with a decrease in pulmonary artery pressure, but rather, with an increase in LV filling pressure that accounts for the decrease in PVR.

(P. 2783 under "Discussion" heading).

Inglessis 2004:

This is a study of 13 patients with an average age of 65 +/- 3 years. (See p. 793 under "Methods" heading). The objective of the study was to see if iNO improved "cardiac performance in patients with RVMI and CS." (See p. 794).

Under the "Methods" heading at p. 794, the reference discloses:

Patients were then included for further study if their right atrial (RA) pressure was >10 mm Hg, their PCWP was no >5 mm Hg higher than the RA pressure, and their CI was <2.5 l/min/m^2. Patients were excluded from the study if they had severe pulmonary edema (PCWP >25 mm Hg; n=4), mechanical complications of MI requiring urgent surgical correction (N=0), severe mitral or aortic valvular disease (n=1), persistent hemodynamically significant tachyarrhythmias (n=1), or a history of clinically significant pulmonary disease (n=0).

The reference further discloses:

In this study, PCWP did not change during NO inhalation by RVMI patients, as has been previously observed during administration to patients with severe LV systolic dysfunction. In patients with severe LV systolic dysfunction, which is usually accompanied by poor diastolic ventricular compliance, breathing NO is thought to increase pulmonary venous return, resulting in an increase in LV filling pressure. The RVMI patients in this study had primarily RV systolic and diastolic function, and the degree of LV dysfunction was not as severe as in

those patients in whom the PCWP has been reported to increase during NO inhalation.

(P. 797, 2nd col.).

Inglessis 2005:

In a reply, the author states "[p]atients with severe LV systolic function should be monitored carefully during chronic NO inhalation because of the possibility of their developing pulmonary venous hypertension." (P. 965, 2nd col.).

Bocchi:

This study included 3 patients ages 40, 41, and 52 years old suffering from either ischemic or idiopathic cardiomyapathy. (P. 70, 1st col.). All three adults had severe pulmonary HTN and refractory heart failure and were candidates for cardiac transplantation. (Id.) All three patients were treated with iNO.

The reference discloses:

> Results of this investigation demonstrate that acute inhaled nitric oxide produces rapid pulmonary vasodilation in the absence of hypoxia in patients with severe heart failure. However, nitric oxide inhalation was associated with an increment in pulmonary pressure, mainly pulmonary wedge pressure, and an improvement in cardiac output. In addition, inhaled nitric oxide may lead to pulmonary edema in patients with severe heart failure.

(P. 71, 1st col.).

Cujec:

This is a case study involving 33 adults with a mean age of 69 +/- 11 years, most of whom had significant valvular disease and dysfunctional LV characterized by a reduced ejection fraction. (P. 816 under "Patients" heading, and p. 819 under "Results" heading).

Cujec concludes at page 823 stating:

> We found in a randomized and blinded trial that the reduction in pulmonary artery systolic pressure following nitric oxide inhalation depends on the pre-existing LVEF. Our results in patients with a broader mix of cardiac pathology confirm previous case series. These observations suggest further limitations for the clinical role of inhaled nitric oxide. We postulate that in patients with the

least cardiac reserve, decreasing venous but not arterial pulmonary vascular resistance may cause an increase in regional pulmonary edema. Through reflex mechanisms, this could further impair cardiopulmonary function resulting in cardiac decompensation, worsening pulmonary hypertension and generalized pulmonary edema. This study cautions against the ubiquitous use of inhaled nitric oxide in the treatment of all critically ill patients. Nitric oxide is not just a pulmonary vasodilator but has profound effects on many other systems. The adverse effects of nitric oxide may become most evident in patients with the least cardiac reserve.

Rosales:

This is a case report of a one-month old neonate that developed rebound pulmonary hypertension after receiving iNO. (See Abstract at p. 224). The infant patient was diagnosed with total anomalous pulmonary venous return (three pulmonary veins draining into the portal system below the diaphragm and the remaining upper left pulmonary vein draining into the innominate vein). (Id.).

This infant underwent surgical correction and in the post operative period received iNO. (See p. 225, 1st col.). iNO was discontinued based on the rationale that the episode of pulmonary HTN may have been caused by left atrial hypertension secondary to a sudden increase in pulmonary blood flow into a non-compliant left atrium and ventricle due in part to the redirection of blood flow from the surgical correction. (See p. 225, 2nd col.).

Argenziano:

This study in pigs resulted in the following conclusion:

In conclusion, we have reproduced, in a porcine model of heart failure and pulmonary hypertension, the constellation of clinically observed hemodynamic responses to inhaled NO therapy, including dose-dependent decreases in pulmonary arterial pressure and PVR and increases in LVEDP. Furthermore, determination of the ESPVR, PRSW, EDPVR, and T in these animals has demonstrated no effect of inhaled NO on myocardial contractility or relaxation. An alternative explanation that has been proposed on theoretical grounds is that volume shifts caused by pulmonary vasodilation are responsible for clinically observed elevations in left atrial pressure and may also explain why patients with preexisting ventricular dysfunction are at greatest risk for these pressure elevations. Although clinical validation of our findings in humans is necessary and is the subject of current investigations, an understanding of this

> mechanism may lead to strategies allowing the safe use of inhaled NO in heart failure, perhaps by adjunctive vasodilator therapy.

(P. 707).

Steinhorn 2007:

This is a review article of persistent pulmonary HTN. It is a general discussion and review, not a clinical study. No data is provided. It points out that iNO is contraindicated in congenital heart disease (e.g., interrupted AO arch, critical AO stenosis, and hypolplastic LV) and severe LV dysfunction.

Under the heading "Treatment with iNO," it states:

> Treatment with iNO for newborns with an OI>25. Nitric oxide (NO) is an endothelial-derived gas signaling molecule that relaxes vascular smooth muscle and that can be delivered to the lung by means of an inhalation device (INOVent; Datex-Ohmeda Ind, Madison, WI).
> In 2 large randomized trials, NO reduced the need for ECMO support by approximately 40%.
> Contraindications to iNO include congenital heart disease characterized by left ventricular outflow tract obstruction (eg, interrupted aortic arch, critical aortic stenosis, hypoplastic left heart syndrome) and severe left ventricular dysfunction.

Krasuski:

This reference reports the results of a clinical study in forty-two adult patients (26 to 77 years old) having pulmonary hypertension during cardiac catheterization and receiving iNO. (See Abstract, p. 2204). The reference concludes that

> Nitric oxide is a safe and effective screening agent for pulmonary vasoreactivity. Regardless of etiology of pulmonary hypertension, pulmonary vasoreactivity is frequently demonstrated with the use of NO. Right ventricular diastolic dysfunction may predict a poor vasodilator response.

(Id. under "Conclusions" heading).

Semigran:

This study included 16 adults (13 men and 3 women) having a mean age of 51 ± 2 years each having class III or IV heart failure and being considered for heart

transplantation. (See p. 983, 1st col.). No patient had a history of primary pulmonary disease, and pulmonary function testing was consistent with chronic left heart failure. (Id.). The patients were treated with digoxin, diuretic drugs, vasodilators and amiodarone. (Id.) iNO was administered at 20, 40 and 80 ppm. (Id. at 2nd col.).

The reference concludes stating:

> Inhaled nitric oxide is a selective pulmonary vasodilator in patients with severe chronic heart failure. The selectivity of inhaled nitric oxide for the pulmonary circulation offers a potential advantage over nonselective vasodilators such as nitroprusside in the identification of reversible pulmonary vasoconstriction in potential heart transplant recipients. Nitric oxide increases left ventricular filling pressure in patients with severe heart failure by an unknown mechanism.

(P. 982 under "Conclusions" heading).

Dickstein:

The reference teaches mathematical (see Appendix at p. 720) and electric circuit (see Figure 1 at p. 717) models of a cardiovascular system as "time varying elastances: the pulmonary and systemic vascular systems were each modeled as a series of resistive and compliance elements." (P. 715 under "Methods" heading).

The reference concludes stating:

> Pulmonary vasodilation by itself can lead to an increase in pulmonary venous pressure that is mediated by shifts of blood between arterial and venous compartments of the pulmonary bed. Furthermore, impairment in ventricular contractile state by itself has relatively little effect on pulmonary venous pressure. The magnitude of the increase in pulmonary venous pressure is largely determined by the volume status and the initial value of pulmonary vascular resistance.

(P. 715 under "Conclusions" heading).

Dickstein further discloses:

> The present analysis suggests that it is not necessary for this agent [i.e., nitric oxide] to work as a negative inotrope to cause pulmonary venous pressure to rise: its pulmonary vasodilating actions alone are sufficient to explain why patients with preexisting heart failure are at greatest risk for pulmonary edema.

(P. 719, 2nd col.).

Henrichsen:

This reference is a letter to the editor of journal reporting iNO treatment of a baby born at 38 weeks of gestation diagnosed with persistent pulmonary hypertension of the newborn (PPHN) and severe left ventricular dysfunction. The baby was treated with 20 ppm iNO which "resulted in an immediate fall in the mean systemic arterial blood pressure from 48 to 35 mm Hg, which reversed when the NO therapy was discontinued." In other words, the iNO caused systemic hypotension.

As second iNO treatment thirty hours later "resulted in a marked improvement in oxygenation, from an arterial oxygen tension to 16 to 420 mm Hg without a change in the systemic arterial blood pressure."

Ovodov:

The review article discusses various clinical studies of PPHN using iNO. (P. 95, 2nd col.). In particular, the reference cites the NINOS trial. (Id.) It concludes that "[s]afety of low-dose inhaled nitric oxide in newborns has been suggested by several studies" and that "there are no reports of any related adverse clinical manifestations." (P. 96, 1st col.).

Adatia:

This reference reports the results of a study involving 11 patients ranging in age from 0.7 to 27 years with a median of 13 years diagnosed with pulmonary hypertension. (P. 1656, 2nd col.). Some of the patients were diagnosed with "severe left ventricular failure despite optimal medical management with digoxin, diuretic drugs and, when appropriate, maximal afterload reduction therapy." (P. 1657, 1st col.).

The reference concludes stating:

> These preliminary observations suggest that nitric oxide is a potent pulmonary vasodilator with minimal systemic effects. It may be useful in discriminating patients needing combined heart and lung transplantation from those requiring exchange of the heart alone.

(P. 1656 under Conclusions heading).

Findlay:

This reference is a case report concerning a 22-year old man treated with iNO where the patient had a "paradoxical response to inhaled nitric oxide, where a rise in mean pulmonary artery and pulmonary artery occlusion pressure and a fall in cardiac output and stroke volume occurred, in a young man with *meningococcaemia*." (P. 134, 1st col.).

Henrichsen is a report of a single near-term neonate having PPHN and LVD that experienced systemic hypotension when treated with iNO, which is contrary to the accepted understanding that is a selective vasodilator, i.e., non-systemic. Moreover, the subsequent iNO treatment had a positive therapeutic outcome. Henrichsen fails to teach LVD as exclusionary criteria in the claimed patient population, and it teaches away from the invention by merely cautioning iNO treatment.

The instant claims are patentable over Ovodov, Adatia and Findlay at least because each reference fails to teach or suggest excluding the claimed patient population having LVD from being treated with iNO.

9(C) Detailed Explanation of Patentability

None of the references disclose excluding from iNO treatment any patient in the patient population (comprising a neonate or near-term neonate) that have been diagnosed as having pre-existing left ventricular dysfunction (LVD) in order to avoid adverse events or serious adverse events. (See independent claims 1, 6, 13 and 17). Thus, independent claims 1, 6, 13 and 17 are patentably novel and nonobvious over the listed most relevant references as well as the other references of record. Moreover,

dependent claims 2-5, 7-12, 14-16 and 18-20 are patentably novel and nonobvious for at least the same reasons set forth herein respecting independent claims 1, 6, 13 and 17.

The AAP reference is highly relevant due to the prominence of the Pediatric Committee. The fact that it is silent respecting excluding from iNO treatment any child patient diagnosed with pre-existing left ventricular function speaks louder than words.

Lipshultz teaches that data and information gleaned from iNO studies in adults do not correlate or are otherwise probative of iNO studies in children. Thus, the Hayward 1996 & 1997, Loh, Inglessis 2004 & 2005, Bocchi, Cujec, Krasuski, Findlay and Semigran references are not probative of the instantly claimed invention.

Pre-existing LVD is not mentioned in the NINOS reference involving infants. While the Roberts involves neonate patients, it fails to teach excluding such patients if they have been diagnosed with pre-existing LVD.

Rosales involves a one-month old neonate patient undergoing surgical correction and post operative iNO treatment. Rosales also fails to teach or suggest pre-existing LVD as exclusionary criteria for iNO treatment.

Argenziano is a pig study that also fails to teach or suggest pre-existing LVD as exclusionary criteria for iNO treatment.

Steinhorn 2007 is a general discussion and review. No data is provided. Therefore, Steinhorn 2007 is a non-enabling reference.

Dickstein is a "purely theoretic analysis of the impact of NO therapy on pulmonary venous pressure." (P. 719, 2nd col.). The reference fails to disclose any data to support this unpredictable science which is also not well understood, therefore, Dickstein is non-enabling prior art. The reference also teaches away from excluding a patient from being treated with iNO where the patient has been diagnosed with pre-existing LVD. For example, the reference theorizes that increased volume causes the risk of adverse events stating:

> results of the present analysis would suggest that patients with heart failure are at increased risk for development of pulmonary edema during NO therapy because of the high effective volume status.

(P. 719, 2nd col.).

Henrichsen is a report of a single near-term neonate having PPHN and LVD that experienced **systemic** hypotension when treated with iNO, which is contrary to the accepted understanding that nitric oxide is a selective vasodilator, i.e., non-systemic. Moreover, the subsequent iNO treatment had a positive therapeutic outcome. Henrichsen fails to teach LVD as exclusionary criteria in the claimed patient population, and it teaches away from the invention by merely cautioning iNO treatment.

The instant claims are patentable over Ovodov, Adatia and Findlay at least because each reference fails to teach or suggest excluding the claimed patient population having LVD from being treated with iNO.

9(D) Concise Statement of Utility

The instantly claimed invention is eligible subject matter under 35 USC 101 for patentable utility in that the claims are generally directed to a method of excluding patients in need of being treated with inhaled nitric oxide. The purpose of such mandatory exclusion is to reduce the incidence of adverse events or serious adverse events. Patients in an intended patient population are excluded from such treatment (even though the inhaled nitric oxide treatment would be potentially beneficial to the patient) if the patient has pre-existing left ventricular dysfunction.

9(E) Showing of Support under 35 USC 112, First Paragraph

Support and antecedent basis for the claimed invention is found at least in the SUMMARY OF THE INVENTION as originally filed at pages 2-4 and ¶¶[0005]-[0020]. Enablement of the claimed invention is found at least in the DETAILED DESCRIPTION OF THE EXEMPLARY EMBODIMENTS at pages 4-13 and ¶¶[0021]-[0050] as well as in EXAMPLE1: INOT22 STUDY at pages 13-22 and ¶¶[0051]-[0069].

9(F) Identification of References Disqualified as Prior Art under 35 USC 103(c)

None of the cited references are disqualified as prior art under 35 USC 103(c).

Respectfully Submitted,

Christopher P. Rogers, Reg. No. 36,334

Date: 21 June 2010

Lee & Hayes, PLLC
601 W. Riverside Avenue, Suite 1400
Spokane, WA 99201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Application Serial Number	TBD
Confirmation Number	TBD
Filing Date	Herein
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Assignee	Ikaria, Inc.
Group Art Unit	TBD
Examiner	TBD
Attorney Docket Number	I001-0002USC3

To: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

From: Christopher P. Rogers (Tel.; Fax 509-323-8979)
Customer Number: 49584
Lee & Hayes, PLLC
601 W. Riverside Avenue, Suite 1400
Spokane, WA 99201

Fees will be paid by credit card through the EFS Web; however the Commissioner is hereby authorized to charge any deficiency of fees and credit any overpayments to Deposit Account Number 12-0769.

Respectfully Submitted,

Dated: 21 June 2010

By: _____
Christopher P. Rogers
Reg. No. 36,334

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE (USPTO)	
Priority Application Serial No	12/494,598
Priority Filing Date	06/30/2006
Title of Application	Methods of Treating Term and Near-Term Neonates Having Hypoxic Respiratory Failure Associated with Clinical or Echocardiographic Evidence of Pulmonary Hypertension
First Named Inventor	James S. Baldassarre
Priority Group Art Unit	1614
Priority Examiner	TBD
Attorney Docket Number	I001-0002USC3

INFORMATION DISCLOSURE STATEMENT

The citations listed are submitted in compliance with the duty of disclosure defined in 37 CFR §1.56. Copies of the cited references were cited or submitted with the priority application and are therefore not submitted herewith.

The Examiner is requested to make these citations of official record in this application.

Respectfully Submitted,

Date: 21 June 2010

By: _____
Christopher P. Rogers
Reg. No. 36,334

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
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	First Named Inventor	James S. Baldassarre
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	Examiner Name	
	Attorney Docket Number	I001-0002USC3

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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	4	WO2010019540;(A1)			2010-02-18	NOVARTIS AG [CH]; PASC		☐

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
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	60	Wessel DL et al., "Improved Oxygenation in a Randomized Trial of Inhaled Nitric Oxide for Persistent Pulmonary Hypertension of the Newborn", PEDIATRICS, Vol. 100, No. 5, 5 Nov 1997.	☐

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EXAMINER SIGNATURE

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement.

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☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

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(A1) The present invention relates to a method for the detection of predisposition to high altitude pulmonary edema (HAPE). It particularly relates to an allelic variants of iNOS (inducible nitric oxide synthase) gene, which has been found to be related with the prevalence of HAPE.

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(71) Applicant *(for all designated States except US)*: **COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH** [IN/IN]; Rafi Marg, 110 001 New Delhi (IN).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PASHA, Abdul, Qadar, Mohammad** [IN/IN]; Institute of Genomics and Integrative Biology, Mall Road, 110 007 New Delhi (IN). **AHSAN, Aarif** [IN/IN]; Institute of Genomics and Integrative Biology, Mall Road, 110 007 New Delhi (IN).

(74) Agent: **BHOLA, Ravi**; K & S Partners, 84-C, C-6 Lane, Sainik Farms, 110 062 New Delhi (IN).

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(54) Title: METHOD OF DETECTING PREDISPOSITION TO HIGH ALTITUDE PULMONARY EDEMA

(57) Abstract: The present invention relates to a method for the detection of predisposition to high altitude pulmonary edema (HAPE). It particularly relates to an allelic variants of iNOS (inducible nitric oxide synthase) gene, which has been found to be related with the prevalence of HAPE.

METHOD OF DETECTING PREDISPOSITION TO HIGH ALTITUDE PULMONARY EDEMA

TECHNICAL FIELD

The present invention relates to a method for the detection of predisposition to high altitude pulmonary edema (HAPE). It particularly relates with the allelic variants of iNOS (inducible nitric oxide synthase) gene, which has been found to be related with the prevalence of HAPE.

BACKGROUND AND PRIOR ART

High altitude pulmonary edema (HAPE) is a form of noncardiogenic pulmonary edema that develops in approximately 10% of randomly selected mountaineers within 24h after rapid ascent to altitude above 4,000 m. A similar phenomenon is observed in the lowlander inductees to a height above 3000 m for various business reasons. An even higher incidence rate of about 60% has been demonstrated in subjects who are susceptible to HAPE as documented by previous occurrence of the disease (Houston CS et al 1960, Bartsch P et al 1997, 1990). HAPE can be effectively prevented by prophylactic use of vasodilators or slow ascent. Nevertheless, it remains the most common cause of death related to high altitude exposure during trekking or mountaineering (Hackett PH et al 1990). The morbidity rate in Himalayan mountaineers was estimated to be 50% if immediate treatment with supplemental oxygen or rapid descent is impossible (Lobenhoffer HP et al 1982). Observed differences in clinical presentations and severity of the disease between racial and ethnic groups together with familial clustering favor a significant hereditary predisposition to the disease.

Although knowledge of the factors influencing the development of HAPE is still incomplete, there is experimental evidence that an exaggerated hypoxic pulmonary vasoconstriction (HPV) plays an important role (Scherrer U et al 1996). An excessive rise in pulmonary artery pressure has been demonstrated by invasive and noninvasive measurements at high altitude in individuals with HAPE. The uneven vasoconstriction in the capillaries sometimes results in "capillary leakage" followed by edema formation (Bartsch P et al 1991). Human subjects who are susceptible to the disease demonstrate an increased pulmonary vascular response even during a brief exposure of high altitude. The underlying pathophysiological mechanism for this exaggerated HPV is still unknown. There is, however, evidence that the endogenous vasodilator nitric oxide (NO) modulates vascular reactivity (Palmer RMJ et al 1987). Regulation of vascular tone by NO is attributed to the intermediates of cGMP pathway (Bellamy TC et al 2002).

The following studies emphasize the involvement of NO in HAPE:

NO exerts its effect mainly via improvement of ventilation/perfusion ratio and lowering of alveolar to arterial oxygen tension difference by increasing arterial oxygen saturation (Scherrer U et al 1996). However, in the healthy volunteers, administration of the NO synthesis antagonist N^G-monomethyl-L-arginine (L-NMMA) during hypoxia increases pulmonary artery pressure and vascular resistance which is similar to that observed in HAPE. Due to this NO has been used as an inhalation therapy for the treatment of HAPE in the affected individuals (Anand IS et al 1998).

Phosphodiesterase 5 is the key enzyme responsible for cGMP hydrolysis in the lungs. The inhibitors of Phosphodiesterase 5 have been found to inhibit hypoxia induced pulmonary hypertension (Goldstein I et al 1998). Hypoxia decreases exhaled NO in mountaineers susceptible to HAPE indicating decreased NO production in such cases (Busch et al 2001). Thus defective NO synthesizing machinery imparting lower NO level may be envisaged to be responsible for the pathogenesis of HAPE. NO is synthesized by three isozymes nNOS (neuronal nitric oxide synthase, NOS1), iNOS (inducible nitric oxide synthase, NOS2) and eNOS (endothelial nitric oxide synthase, NOS3) (Michel T et al 1997). NOS1 and NOS3 are constitutively expressed while NOS2 is expressed upon induction. Among these the best candidate which is supposed to be defective in HAPE is eNOS (endothelial nitric oxide synthase) while induction of iNOS (inducible nitric oxide synthase) seems to be inevitable for the immediate recovery of the total NO reserve (Xia Y et al 1998). Moreover, robust cell signaling mechanisms generally favor the recruitment of inducible genes for immediate early physiological responses. It can be speculated that a defect in iNOS which doesnot permit its activation may not recover the reduced NO level in individuals exposed to hypoxia resulting in HAPE.

The defect in iNOS may occur at genetic level in HAPE patients. In numerous cases, the expression of the genes has been found to get altered by the polymorphisms in the gene sequence (Qadar Pasha MA et al 2001). Hence, it is always possible that polymorphism in iNOS gene may alter its

expression and associates with the disease.

Current status of the treatment of HAPE:

1. NO therapy: NO is being used as an inhalation therapy for the treatment of HAPE. It exerts its effect mainly via improvement of ventilation/perfusion ratio and lowering of alveolar to arterial oxygen tension difference by increasing arterial oxygen saturation.

NO induced improvement in arterial oxygenation in subjects with HAPE was accompanied

by a shift in blood flow in the lung away from edematous segments and toward nonedematous segments results in evening/homogeneity of the vasoconstriction throughout the capillaries (Scherrer U et al 1996, Anand IS et al 1998).

2. Rapid descent: Rapid descent of HAPE patients not only prevents the worsening but even improves the pathogenesis of the disease (Hackett PH et al 2001).

3. Portable Air Chambers (PACs): PACs in the form of small cylinders filled with oxygen is often used as inhalation therapy for HAPE (Hackett PH et al 2001).

4. Genetic predisposition: The only study in this context suggests that genetic variation in endothelial nitric oxide synthase gene (eNOS) and angiotensin converting enzyme gene (ACE) may predispose individuals to HAPE (Droma Y et al 2002). The results are as follows:

	Controls	Patients
Glu298Asp (eNOS)	9.8%	25.6%
B/A (eNOS)	6.9%	32.2%
I/D (ACE)	4%	22%

Limitations of the available therapies for HAPE:

1. HAPE patients do not found to have homogenous response to NO inhalation. Moreover, concentration of required NO varies with the severity of the disease. Sometimes inadequate inhalation results in hypotension or even septic shock to the patients.
2. Immediate descent of the HAPE patients often remains impossible due to severe weather and rugged terrain (Anand IS et al 1998, Hackett PH et al 2001).
3. Carriage of PACs sometimes appears to be not feasible due to overloading problem. Improved conditions of the disease are often temporary as removal of chambers renders the patient worse (Hackett PH et al 2001).
4. The reported polymorphisms associated with HAPE are not specific but have also been shown to be associated with the disorders like diabetes, coronary artery disease, hypertension and myocardial infarction where elevated blood pressure is observed

(Monti LD et al 2003, Via M et al 2003). The allelic frequency difference mentioned appears to be the same with other diseases. Hence the possibility of allelic contribution to the disease may be due to other related pathophysiologies like hypertension, which involves the exacerbations of HAPE. Moreover, the study does not include HA natives (high landers), a population residing blissfully in the same environment where the disease occurs.

Novelty of the invention is in providing a novel method for the detection of predisposition to HAPE.

Still another novelty is for providing a novel marker region in iNOS gene.

Still another novelty is for providing a novel SNP in iNOS gene.

Still another novelty is to demonstrate association of the allelic variants of iNOS gene with HAPE.

Another novelty is to provide novel primers and probes for amplification, which contains the novel SNP.

OBJECTS OF THE INVENTION:

Main object of the present invention is to provide a method for the detection of predisposition to HAPE, which obviates the limitations listed above.

Still another object is providing a novel SNP in iNOS gene.

Another object is to provide novel primers and probes for amplification, which contains the novel SNP.

Another object is to perform association analysis for the allelic variants between low landers and HAPE patients so that the relation with the disease could be scored.

SUMMARY OF THE INVENTION:

The present invention relates to the method of detection of predisposition to HAPE. It particularly relates with the allelic variants of iNOS gene, which has been related to the prevalence of HAPE. Defective Nitric Oxide (NO) synthesizing machinery imparting lower NO level has been envisaged to be responsible for the pathogenesis of HAPE. iNOS gene has been shown to be responsible for NO production as the inhibitors of NO production increased the severity of HAPE. Present invention provides a method for detection of predisposition to HAPE as the novel allelic variants of iNOS gene in the disclosed marker region was shown to be negatively associated with the prevalence of HAPE in a population.

BRIEF DESCRIPTION OF ACCOMPANYING FIGURES/DRAWINGS

Figure 1 Schematic representation of the gene of inducible Nitric Oxide Synthase

(iNOS) localization: 17 cenq[11.2]. The vertical bars showing the exonic regions (From Gene bank Nucelotide Sequence ID No. NT_010799).

Figure 2 shows sequence file of the individual with AA homozygote.

Figure 3 shows sequence file of the individual with GG homozygote.

Figure 4 shows sequence file of the individual with AG heterozygote.

Figure 5 shows sequence file of the individual with TC heterozygote.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the preferred embodiments of the invention given for the purpose of disclosure. Alternative embodiments of the invention can be envisaged by those skilled in the art. All such alternative embodiments are intended to lie within the scope of this invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the method of detection of predisposition to HAPE. It particularly relates with the allelic variants of iNOS gene, which has been found to be related to the prevalence of HAPE.

I. Identification of the marker region on the iNOS gene:

Taking in consideration the important functions of NO at high altitude, iNOS, the inducible nitric oxide synthase gene was selected as the candidate gene for the study.

II. Selection of the study subjects:

Clinical severity of HAPE was assessed by Lake Louise acute mountain sickness (AMS) scoring system. Briefly, the patients were assessed for the presence of five symptoms: headache, gastrointestinal upset, fatigue, weakness, or both, dizziness, lightheadedness, or both, and difficulty in sleeping. Change in mental status, ataxia and peripheral edema were also assessed. Each of these symptoms were rated between 0 and 3. A score of 0 indicated no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. HAPE score is the sum of all 8 symptoms and patients were characterized by HAPE score>6 (Anand IS et al 1998). Lowlanders (LLs) were subjects who even after induction to high altitudes atleast thrice never found to have any of the above mentioned symptoms. High altitude (HA) natives were the permanent residents of HA from ancient times.

III. Extraction of genomic DNA from leukocytes:

Genomic DNA was extracted from blood using salting out method. Lysis of red blood cells in presence of high salt was followed by treatment with Nucleus lysis buffer (NLB). Proteins were precipitated and extraction of DNA was obtained in ethanol (Miller SA et al 1988).

IV. Identification of the allelic variants of the iNOS gene:

Novel polymorphism of the invention:

As a first step to the present invention, the applicants carried out the PCR amplification of marker region of the iNOS gene using self designed oligonucleotide primers. The primers were designed in accordance with the human iNOS gene sequence (Gene Bank Accession Number NT_010799). The sequencing of the purified PCR product revealed a novel single nucleotide polymorphism in Intron 7 of the human iNOS gene. It was apparent, therefore that there is a hitherto unrecognized allele or subtype of the human iNOS gene.

The present invention provides a sequence for the allelic variants of human iNOS gene comprising the following novel single nucleotide polymorphism compared with the human iNOS gene sequence in the database.

For example, the nucleotide sequence of the allelic variant of human iNOS gene (SEQ ID NO: 1) having the polymorphic site listed in Table 1 may be-

5'CAGCGGAGTGATGGCAAGCACGACTTCCGGGTGTGGAATGCTCAGCT
CATCCGCTATGCTGGCTACCAGATGCCAGATGGCAGCATCAGAGGGGA
CCCTGCCAACGTGGAATTCACTCAGGTACCCGGCCCAGCCTCAGCC
A*/GCCGGCCATTGGGGCGGGGAGCCCCGTGGTGAGCGAGTGACAGAGT
GGAGCCCAGAGGAGACACGCAGCCCGGGCTTACAGACTCACAGGGCCC
GTCTTGTTCCCCAGCTGTGCATC3'

In the above sequence the SNP* is shown in bold.

Table 1

Site of change	Base change	Mutation type
19480	A/G	Transition

V. Association Analysis with the disease

Analysis of the SNP in 42 HA natives, 39 HAPE controls and 18 HAPE patients revealed three genotypes, namely AA, AG and GG. The distribution of alleles is summarized in Table 2.

Table 2

Study subjects	A	G
HAPE controls (n=39)	0.35	0.65
HAPE patients (n=18)	0.58	0.42
HA natives (n=42)	0.18	0.82

The frequency of the G allele was found to be in the order of HA natives>HAPE controls>HAPE subjects. The biostatistical analysis showed a significant association of G allele with HA adaptation and A allele with the disease as mentioned in Table 3.
Herein the odds ratio (OR) and 95% confidence of interval was used as a measure of the strength of the association between genotypic combination and the disease. P value of <0.05 was considered statistically significant.

Table 3

Association type	χ^2 value	p value	Odds ratio	95% CI	Relative risk
HAPE patients & HAPE controls	10.63	0.001	2.56	1.45-4.54	1.66 (1.21-2.27)
HAPE patients & HA natives	33.96	<0.001	6.29	3.30-12.01	3.22 (2.05-5.06)
HAPE controls & HA natives	7.42	0.006	-	-	-

Nitric oxide synthase for its reaction to synthesize nitric oxide, requires oxygen which acts as a cofactor in the reaction. Oxygen binds to the oxygenase domain in iNOS and contributes to the synthesis of NO. In hypoxic condition scarcity of oxygen may lead to lower NO production, however any modification in the oxygenase domain, which modify the activity of the enzyme in such a way that it requires no oxygen or less oxygen may contribute to normal NO production. NO improves oxygenation of hemoglobin and normal NO production may involve the mechanisms acting in acclimatization, hence any alteration in oxygenase domain may be favorable for the production of NO. In the present investigation the novel SNP found in intron 7 is present near to the oxygenase domain of NOS2 gene which spans exon 7 to exon 16. It is quite possible that the SNP found is in

linkage disequilibrium to a nearby SNP, which is contributing to the final impact on NO production by NOS2 gene.

VI. Diagnostic kits

The invention further provides diagnostic kit comprising at least one or more allele specific oligonucleotides as described in SEQ ID 2 and 3. Often, the kits contain one or more pairs of allele-specific oligonucleotides hybridizing to different forms of a polymorphism. In some kits, the allele-specific oligonucleotides are provided immobilized to a substrate. For example, the same substrate can comprise allele-specific oligonucleotide probes for detecting at least the polymorphism shown in Table1. Optional additional components of the kit include, for example, restriction enzymes, reverse transcriptase or polymerase, the substrate nucleoside triphosphates, means used to label (for example, an avidin enzyme conjugate and enzyme substrate and chromogen if the label is biotin), and the appropriate buffers for reverse transcription, PCR, or hybridization reactions. Usually, the kit also contains instructions for carrying out the methods.

VII. Nucleic acid vectors

Variant genes can be expressed in an expression vector in which a variant gene is operably linked to a native or other promoter. Usually, the promoter is eukaryotic promoter for expression in a mammalian cell. The transcription regulation sequences typically include a heterologous promoter and optionally an enhancer, which is recognized by the host. The selection of an appropriate promoter, for example trp, lac, phage promoters, glycolytic enzyme promoters and tRNA promoters, depends on the host selected. Commercially available expression vectors can also be used. Suitable host cells include bacteria such as E.coli, yeast, filamentous fungi, insect cells, mammalian cells, typically immortalized, e.g., mouse, CHO, human and monkey cell lines and derivatives thereof. Preferred host cells are able to process the variant gene product to produce an appropriate mature polypeptide.

The invention further provides transgenic non-human animals capable of expressing an exogenous variant gene and/or having achieved by operably linking the gene to a promoter and optionally an enhancer, and microinjecting the construct into a zygote. Inactivation of endogenous variant genes can be achieved by forming a transgene in which a cloned variant gene is inactivated by insertion of a positive selection marker. The transgene is then introduced in to an embryonic stem cell, where it undergoes homologous recombination with an endogenous variant gene. Mice and other rodents are preferred animals. Such animals provide useful drug screening systems.

Accordingly, the main embodiment of the present invention relates to a method for

detecting predisposition to high altitude pulmonary edema (HAPE), said method comprising the steps of:

(a) selecting study subjects by monitoring high altitude pulmonary edema associated symptoms,

(b) extracting genomic DNA from leukocytes by conventional methods from the study subjects,

(c) amplifying Intron 7 of the human iNOS gene of SEQ ID No.1 by designing and synthesizing Forward and Reverse oligonucleotide primers of SEQ ID No. 2 and SEQ ID No. 3, respectively,

(d) identifying computationally novel Single Nucleotide Polymorphism (SNP) by comparing with the already existing sequence of human iNOS gene,

(e) screening the high altitude native population (HA natives), low lander natives (HAPE controls) and low lander HAPE patients for the novel single nucleotide polymorphism, using above said primers of SEQ ID No. 2 (Forward Primer) and SEQ ID 3 (Reverse Primer),

(f) computing the frequencies of AA, AG and GG genotypes in the populations of step (e) for establishing the association of the genotypes with high altitude pulmonary edema, and

(g) predicting and statistically analyzing the differences in the distribution of the allelic variants (AA, AG and GG genotypes) in the populations wherein GG genotype at 19480 position are at low risk to high altitude pulmonary edema and AA genotype at 19480 position are at high risk of the disease.

Another embodiment of the present invention relates to the oligonucleotide primers capable for amplification of Intron 7 of human iNOS gene are selected from group comprising of

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer, and

(b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

Yet another embodiment of the present invention relates to the oligonucleotide primers contain one or more polymorphic sites selected group comprising of

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer, and

(b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer.

Still another embodiment of the present invention relates to the allelic variants wherein the allelic variants of the of iNOS gene have AA, AG and GG genotypes

A diagnostic kit for the detection of SNP genotypes having predisposition to high altitude pulmonary edema (HAPE) said kit comprising of primers and probes:

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer

(b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

One more embodiment of the present invention relates to the Primers suitable for amplification of iNOS gene region containing one or more polymorphic sites, said primers include:

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer

(b) SEQ ID 3: 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

In another embodiment of the present invention relates to the nucleic acid vectors containing the allelic variants of the iNOS gene.

The following examples are given by way of illustration of the present invention and should not be construed to limit the scope of the present invention.

EXAMPLES

EXAMPLE 1

Identification of the marker gene:

Taking in consideration the important functions of NO at HA, iNOS, the inducible nitric oxide synthase was selected as the candidate gene for the study.

EXAMPLE 2

Selection of the study subjects:

Clinical severity of HAPE was assessed by Lake Louise acute mountain sickness (AMS) scoring system. Briefly, the patients were assessed for the presence of five symptoms: headache, gastrointestinal upset, fatigue, weakness, or both, dizziness, lightheadedness, or both, and difficulty in sleeping. Change in mental status, ataxia and peripheral edema were also assessed. Each of these symptoms were rated between 0 and 3. A score of 0 indicated no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. HAPE

score is the sum of all 8 symptoms and patients were characterized by HAPE score>6 (Anand IS et al 1998). LLs were subjects who even after induction to high altitudes atleast thrice never found to have any of the above mentioned symptoms. HA natives were the permanent residents of HA from ancient times.

EXAMPLE 3

Extraction of genomic DNA from leukocytes:

Genomic DNA was extracted from blood using salting out method. Lysis of red blood cells in presence of high salt was followed by treatment with Nucleus lysis buffer (NLB). Proteins were precipitated and DNA was extracted from peripheral blood leukocytes using a modification of the salting out procedure. The concentration of the DNA was determined by measuring the optical density of the sample, at a wavelength of 260 nm. (Miller SA et al 1988).

EXAMPLE 4

Identification of the allelic variants of the iNOS gene:

This example describes the identification of allelic variants of iNOS gene by PCR and sequencing using certain oligonucleotide primers according to the invention. The DNA was then amplified by polymerase chain reaction by using the oligonucleotide primers:

1. 5'CAG CGG AGT GAT GGC AAG CAC GAC 3'(as listed in SEQ ID NO:2) and
2. 5' GAT GCA CAG CTG GGG AAC AAG ACG 3'(as listed in SEQ ID NO:3).

Polymerase chain reaction was carried out using the following conditions:

Step 1 94^0C for 4 min

Step 2 94^0C for 30 sec

Step 3 62.5^0C for 30 sec

Step 4 72^0C for 45 sec

Step 5 34 times to Step 2

Step 6 72^0C for 10 min

PCR was performed in a Perkin Elmer GeneAmp PCR System 9600. This reaction produced a DNA fragment of 258bp when analyzed by 2% agarose gel electrophoresis. The PCR product was purified from band cut out of agarose gel using a Amersham Pharmacia gel extraction kit (Amersham) and both the strands of the PCR product were directly sequenced using dye terminator chemistry on an ABI Prism 377 automated DNA sequencer. The PCR product was identical to the human iNOS gene sequence except of the novel single base pair change mentioned in Table1.

EXAMPLE 5

Nucleotide sequence of the Allelic Variant of the iNOS gene:

The nucleotide sequence of the allelic variant of iNOS gene derived using the method as described in example 1 -

5'CAG CGG AGT GAT GGC AAG CAC GAC TTC CGG GTG TGG AAT GCT CAG CTC ATC CGC TAT GCT GGC TAC CAG ATG CCA GAT GGC AGC ATC AGA GGG GAC CCT GCC AAC GTG GAA TTC ACT CAG GTA CCC GGC CCA GCC TCA GCC **A*/G**CC GGC CAT TGG GGC GGG GAG CCC CGT GGT GAG CGA GTG ACA GAG TGG AGC CCA GAG GAG ACA CGC AGC CCG GGC TTA CAG ACT CAC AGG GCC CGT CTT GTT CCC CAG CTG TGC ATC 3'

In the above sequence the SNP* is shown in bold.

EXAMPLE 6

G allele is related with adaptation and A allele associates with the disease:

A method as described in example 4 is applied to a series of DNA samples extracted from HA natives, HAPE controls and HAPE patients. A highly significant association of G allele with the HA adaptation and A allele with the disease has been observed. The results are summarized in the table below:

Association type	χ^2 value	p value	Odds ratio	95% CI	Relative risk
HAPE patients & HAPE controls	10.63	0.001	2.56	1.45-4.54	1.66 (1.21-2.27)
HAPE patients & HA natives	33.96	<0.001	6.29	3.30-12.01	3.22 (2.05-5.06)
HAPE controls & HA natives	7.42	0.006	-	-	-

Hence, individuals with GG genotype being at low risk and those with AA genotype being at high risk for HAPE, can be expected to hold true for other populations also.

EXAMPLE 7

Nucleic acid vectors containing the iNOS variant sequences:

Vectors and host cells transformed with the allelic variants of the iNOS gene containing one or more polymorphic sites as listed in table 1, can be prepared, for example, as detailed

below.

Variant genes can be expressed in an expression vector in which a variant gene is operably linked to a native or other promoter. Usually, the promoter is eukaryotic promoter for expression in a mammalian cell. The transcription regulation sequences typically include a heterologous promoter and optionally an enhancer, which is recognized by the host. The selection of an appropriate promoter, for example trp, lac, phage, glycolytic enzyme and tRNA, depends on the host selected. Commercially available expression vectors can also be used. Suitable host cells include bacteria such as E.coli, yeast, filamentous fungi, insect cells, mammalian cells, typically immortalized, e.g., mouse, CHO, human and monkey cell lines and derivatives thereof. Preferred host cells are able to process the variant gene product to produce an appropriate mature polypeptide.

Advantages of the present invention:

The present invention adds following points to the treatment of HAPE.

1. Inducible nitric oxide synthase gene as a novel marker for HAPE studies.
2. Novel primer sequences responsible for the amplification of PCR product containing novel SNP.
3. Novel SNP (19480 A/G) that can be used for further association studies.
4. A significant association of wild type allele (A) to the disease (Table 2 and 3).
5. A significant association of mutant allele (G) to adaptation (Table 2 and 3).
6. A significant difference between the frequency of alleles with respect to HA native and HAPE controls (Table 2 and 3).
7. The presence of G allele predisposes an individual to less chances of getting diseased.
8. It may help individuals to decide visiting high altitude for various reasons.

Provided below is the sequence listing information for SEQ ID Nos. 1, 2 and 3

SEQUENCE LISTING

GENERAL INFORMATION

APPLICANT: CSIR

TITLE OF INVENTION: Method for the detection of predisposition to high altitude pulmonary edema (HAPE).

NUMBER OF SEQUENCES: 03

CORRESPONDING ADDRESS: Institute of genomics and integrative biology, CSIR, Delhi University Campus, Mall Road-110007, India.

Telephone: +91-11-27666156 Fax: +91-11-27667471

INFORMATION FOR SEQUENCE ID NO: 1

1. SEQUENCE CHARACTERISTICS:

1. LENGTH: 258 bp

2. TYPE: DNA

5'CAG CGG AGT GAT GGC AAG CAC GAC TTC CGG GTG TGG AAT GCT CAG CTC ATC CGC TAT GCT GGC TAC CAG ATG CCA GAT GGC AGC ATC AGA GGG GAC CCT GCC AAC GTG GAA TTC ACT CAG GTA CCC GGC CCA GCC TCA GCC **A*/G**CC GGC CAT TGG GGC GGG GAG CCC CGT GGT GAG CGA GTG ACA GAG TGG AGC CCA GAG GAG ACA CGC AGC CCG GGC TTA CAG ACT CAC AGG GCC CGT CTT GTT CCC CAG CTG TGC ATC 3'

3. ORGANISM: *Homo sapiens* (Humans)

4. IMMEDIATE SOURCE: PCR

5. NAME/KEY: Marker Region

6. SEQUENCE ID # 1

INFORMATION FOR SEQUENCE ID NO: 2

1. SEQUENCE CHARACTERISTICS:

LENGTH: 24 bp

TYPE: DNA

5'CAG CGG AGT GAT GGC AAG CAC GAC 3'

ORGANISM: Artificial sequence

IMMEDIATE SOURCE: Synthetic

NAME/KEY: Synthetic Oligonucleotide

SEQUENCE ID # 2

INFORMATION FOR SEQUENCE ID NO: 3

1. SEQUENCE CHARACTERISTICS

LENGTH: 24 bp

TYPE: DNA

5' GAT GCA CAG CTG GGG AAC AAG ACG 3'

ORGANISM: Artificial sequence

IMMEDIATE SOURCE: Synthetic

NAME/KEY: Synthetic Oligonucleotide

SEQUENCE ID # 3

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Synthase Gene Polymorphisms With High-Altitude Pulmonary Edema.Circulation 2002;106: 826-830.

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20. Miller, S.A., Dykes, D.D et al. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1988; 16: 1215.

We claim:

1. A method for detecting predisposition to high altitude pulmonary edema (HAPE), said method comprising the steps of:

   (a) selecting study subjects by monitoring high altitude pulmonary edema associated symptoms,

   (b) extracting genomic DNA from leukocytes by conventional methods from the study subjects,

   (c) amplifying Intron 7 of the human iNOS gene of SEQ ID No.1 by designing and synthesizing Forward and Reverse oligonucleotide primers of SEQ ID No. 2 and SEQ ID No. 3, respectively,

   (d) identifying computationally the Novel Single Nucleotide Polymorphism (SNP) by comparing with the already existing sequence of human iNOS gene,

   (e) screening the high altitude native population (HA natives), low lander natives (HAPE controls) and low lander HAPE patients for the novel single nucleotide polymorphism, using above said primers of SEQ ID No. 2 (Forward Primer) and SEQ ID 3 (Reverse Primer),

   (f) computing the frequencies of AA, AG and GG genotypes in the populations of step (d) for establishing the association of the genotypes with high altitude pulmonary edema, and

   (g) predicting and statistically analyzing differences in the distribution of the allelic variants (AA, AG and GG genotypes) in the populations and wherein GG genotype at 19480 position are at low risk to high altitude pulmonary edema and AA genotype at 19480 position are at high risk to of the high altitude pulmonary edema.

2. A method as claimed in claim 1 wherein, the oligonucleotide primers capable for amplification of Intron 7 of human iNOS gene are selected from group

   (a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer, and

   (b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

3. A method as claimed in claim 3 wherein, the oligonucleotide primers contain one

or more polymorphic sites selected group comprising of:

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer, and

(b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer.

4. A method as claimed in claim 1 wherein, the allelic variants of iNOS gene have AA, AG and GG genotypes.

5. A diagnostic kit for the detection of SNP genotypes having predisposition to high altitude pulmonary edema (HAPE) said kit comprising of primers and probes:

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer

(b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

6. A pair of primers suitable for amplification of iNOS gene region containing one or more polymorphic sites, said primers include:

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer

(b) SEQ ID 3: 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

7. The nucleic acid vectors containing the allelic variants of the iNOS gene.

AMENDED CLAIMS
[received by the International Bureau on 31 August 2004 (31.08.2004);
original claims 1-7 replaced by new claims 1-6 (2 pages)]

1. A method for detecting predisposition to high altitude pulmonary edema (HAPE), said method comprising the steps of:

   (a) selecting study subjects by monitoring high altitude pulmonary edema associated symptoms,
   (b) extracting genomic DNA from leukocytes by conventional methods from the study subjects,
   (c) amplifying Intron 7 of the human iNOS gene of SEQ ID No.1 by designing and synthesizing Forward and Reverse oligonucleotide primers of SEQ ID No. 2 and SEQ ID No. 3, respectively,
   (d) identifying computationally the Novel Single Nucleotide Polymorphism (SNP) by comparing with the already existing sequence of human iNOS gene,
   (e) screening the high altitude native population (HA natives), low lander natives (HAPE controls) and low lander HAPE patients for the novel single nucleotide polymorphism, using above said primers of SEQ ID No. 2 (Forward Primer) and SEQ ID 3 (Reverse Primer),
   (f) computing the frequencies of AA, AG and GG genotypes in the populations of step (d) for establishing the association of the genotypes with high altitude pulmonary edema, and
   (g) predicting and statistically analyzing differences in the distribution of the allelic variants (AA, AG and GG genotypes) in the populations and wherein GG genotype at 19480 position are at low risk to high altitude pulmonary edema and AA genotype at 19480 position are at high risk to of the high altitude pulmonary edema.

2. A method as claimed in claim 1 wherein, the oligonucleotide primers capable for amplification of Intron 7 of human iNOS gene are selected from group

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer, and

(b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

3. A method as claimed in claim 2 wherein, the oligonucleotide primers identify one or polymorphic sites responsible for HAPE.

4. A method as claimed in claim 1 wherein, the allelic variants of iNOS gene have AA, AG and GG genotypes

5. A diagnostic kit for the detection of SNP genotypes having predisposition to high altitude pulmonary edema (HAPE) said kit comprising of primers and probes:

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer

(b) 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

6. A pair of primers suitable for amplification of iNOS gene region containing one or more polymorphic sites, said primers include

(a) 5' CAG CGG AGT GAT GGC AAG CAC GAC 3' (SEQ ID No.2), which is a forward primer

(b) SEQ ID 3: 5' GAT GCA CAG CTG GGG AAC AAG ACG 3' (SEQ ID No.3), which is a reverse primer

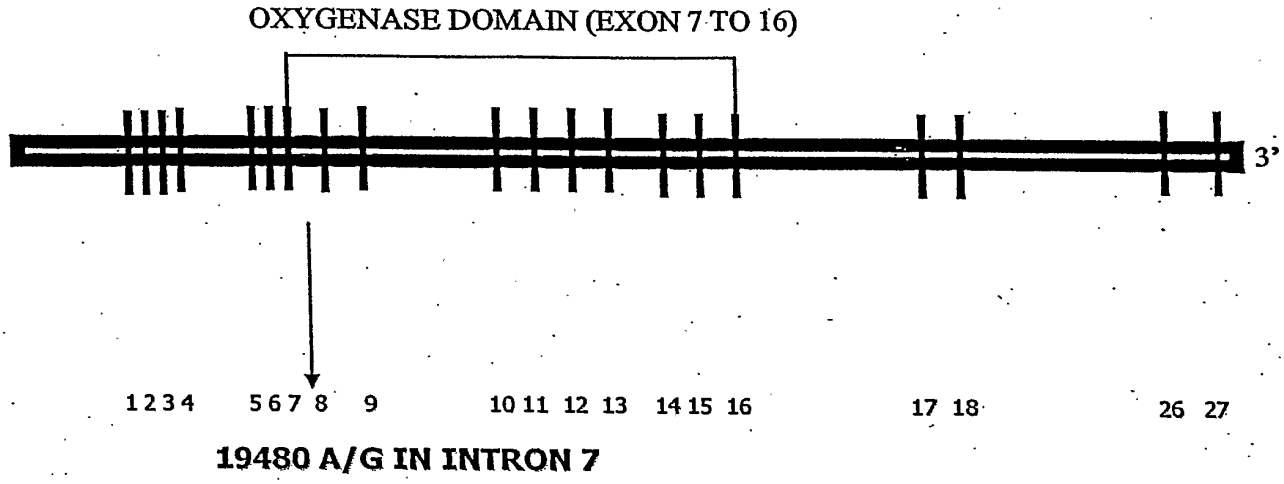
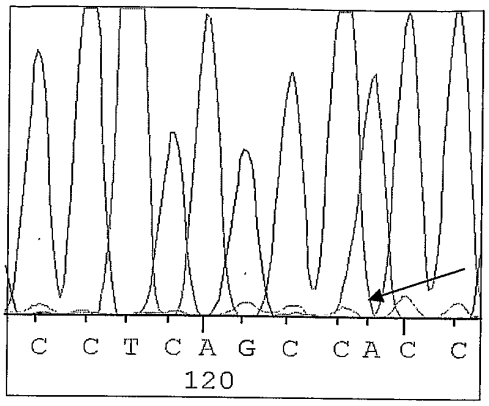
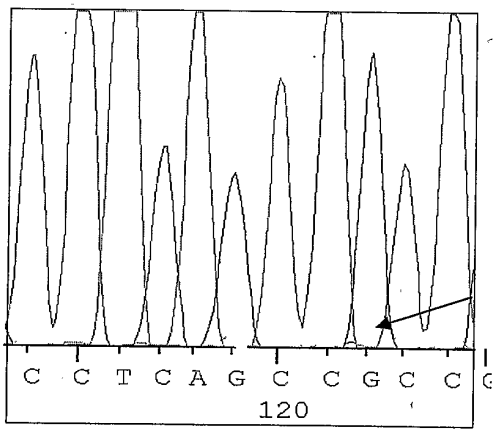

OXYGENASE DOMAIN (EXON 7 TO 16)
3'
1 2 3 4
5 6 7 8
9
10 11 12 13
14 15 16
17 18
26 27
19480 A/G IN INTRON 7

Fig. 1



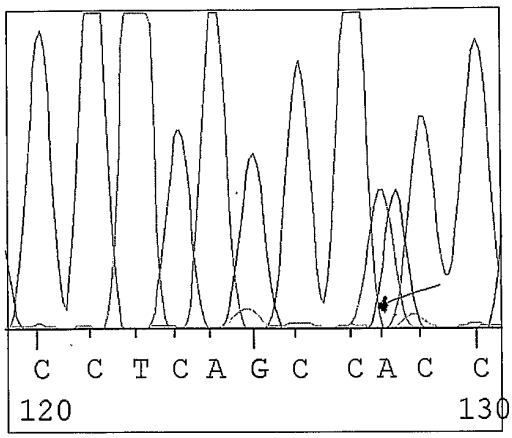
19480 AA

Figure 2



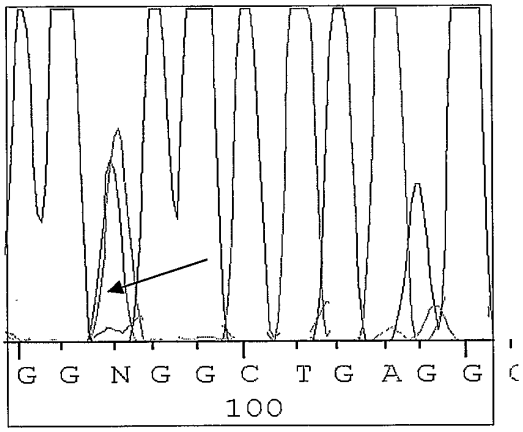
19480 GG

Figure 3



19840 AG

Figure 4



19480 TC

Figure 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 03/05158

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, BIOSIS, WPI Data, EMBASE, SEQUENCE SEARCH, PAJ, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! EBI; Retrieved on 04.06.2004, "Alignment display for SEQ ID NO:1" retrieved from EBI Database accession no. AC131306 XP002283507	7
A	abstract	1-6

A	DATABASE GENBANK 'Online! Partial sequence, 19 February 1904 (1904-02-19) "H.sapiens, chromosome 17, genomic contig" retrieved from NCBI Database accession no. NT_010799 XP002283508 abstract	1-7

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 2004	26/07/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bradbrook, D

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BASNYAT B ET AL: "High-altitude illness" LANCET THE, LANCET LIMITED. LONDON, GB, vol. 361, no. 9373, 7 June 2003 (2003-06-07), pages 1967-1974, XP004429770 ISSN: 0140-6736 page 1971, column 2, paragraph 4 abstract	1-7
A	DROMA YUNDEN ET AL: "Positive association of the endothelial nitric oxide synthase gene polymorphisms with high-altitude pulmonary edema" CIRCULATION, vol. 106, no. 7, 13 August 2002 (2002-08-13), pages 826-830, XP002283504 ISSN: 0009-7322 cited in the application abstract	1-7
A	WEISS JOHANNA ET AL: "Lack of evidence for association of high altitude pulmonary edema and polymorphisms of the NO pathway." HIGH ALTITUDE MEDICINE & BIOLOGY. UNITED STATES 2003 FALL, vol. 4, no. 3, October 2003 (2003-10), pages 355-366, XP001181946 ISSN: 1527-0297 abstract	1-7
A	XU WEIMING ET AL: "Molecular cloning and structural organization of the human inducible nitric oxide synthase gene (NOS2)" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 219, no. 3, 1996, pages 784-788, XP002283505 ISSN: 0006-291X figure 1; table 1 -& DATABASE GENBANK 'Online! H.sapiens NOS2 gene, exons 8 and 9, 19 August 1996 (1996-08-19) retrieved from NCBI Database accession no. X85766 XP002283548 abstract	1-7

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 03/05158

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHARTRAIN NICOLE A ET AL: "Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 9, 1994, pages 6765-6772, XP002283506 ISSN: 0021-9258 figure 1; table 1 ---	1-7
A	DATABASE SNP 'Online! SNP in iNOS gene at pos. 845034 of NT_010799, 11 May 2003 (2003-05-11) retrieved from NCBI Database accession no. RS2297520 XP002283509 abstract -----	1-7

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 03/05158

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-4 (in part)
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.: 1-4,7 (in part)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 1-4 encompass a surgical step carried out on the human/animal body, the search has been carried out assuming the absence of such a step.

Continuation of Box I.1

Claims Nos.: 1-4 (in part)

Rule 39.1(iv) PCT - Method of surgery on the human or animal body (Claims 1-4)

Continuation of Box I.2

Claims Nos.: 1-4,7 (in part)

Claims 1-4 are unclear contrary to the requirements of Art.6 PCT for the following reasons:

Claims 1-4 are directed to a method for detecting a predisposition to HAPE according to the genotype of an individual at a particular polymorphic site in the iNOS gene. According to the application, said polymorphism is in intron 7 of the iNOS gene, at position 19480 (cf p.6, l.10-11 and Table 1). However, said definition does not unambiguously identify the polymorphic site: no indication is given as to how the stated position relates to any disclosed nucleotide sequence for the iNOS gene. Separate reference is made to the contiguous genomic sequence with Gene Bank Accession Number NT_010799 (cf Fig.1), wherein the position of the iNOS gene is between bases 820786 and 864549. The sequence defined by SEQ ID NO.1 contains the polymorphism (cf p.6, l.16-23): a search using this sequence provided matches with GenBank database sequences AC131306, AL354047 and AC130289 (cf D1: sequence alignments), which are genomic clones from human chromosome 17, and which do not indicate the position of the iNOS gene. A 94.6% match was also found with sequence X85766, with the polymorphism located at position 300, i.e. in intron 8 of the iNOS gene (cf D1 and D6: Xu et al and X85766). This corresponds with position 845034 of NT_010799 (cf D2: NT_010799 partial sequence).

Therefore, this is taken as being the position of the polymorphism, and search was based on this polymorphic sites an SEQ ID NO.1.

Claim 3 is unclear (Art.6 PCT) in that it refers to the primers containing one or more polymorphic sites, yet these have not been defined. It is noted that neither of the primers would hybridize across the polymorphic site of interest. Therefore, claim 3 was searched only insofar as it relates to the primer sequences defined by SEQ ID NOs 2 and 3.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claim 7 is unclear (Art.6 PCT) in that it refers to "The nucleic acid vectors containing the allelic variants of the iNOS gene". It is unclear what vectors and what allelic variants are being referred to. As the only variant referred to in the application is that discussed above, claim 7 was searched with respect to any vector comprising SEQ ID NO.1 or the polymorphism defined above.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

WO2005004884

Publication Title:

TREATMENT OF SPECIFIC CARDIOVASCULAR CONDITIONS WITH NITRITE

Abstract:

Abstract of WO2005004884

It has been surprisingly discovered that administration of nitrite to subjects causes a reduction in blood pressure and an increase in blood flow to tissues. The effect is particularly beneficial, for example, to tissues in regions of low oxygen tension. This discovery provides useful treatments to regulate a subject's blood pressure and blood flow, for example, by the administration of nitrite salts. Provided herein are methods of administering a pharmaceutically-acceptable nitrite salt to a subject, for treating, preventing or ameliorating a condition selected from : (a) ischemia-reperfusion injury (e.g., hepatic or cardiac or brain ischemia-reperfusion injury); (b) pulmonary hypertension (e.g., neonatal pulmonary hypertension); or (c) cerebral artery vasospasm. Data supplied from the esp@cenet database - Worldwide c70

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 January 2005 (20.01.2005)

PCT

(10) International Publication Number
WO 2005/004884 A2

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(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/485,959 9 July 2003 (09.07.2003) US
60/511,244 14 October 2003 (14.10.2003) US

(71) Applicants *(for all designated States except US)*: **THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; National Institutes of Health, Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US). **THE BOARD OF SUPERVISORS OF LOUISIANA STATE UNIVERSITY AND AGRICULTURAL AND MECHANICAL COLLEGE, ACTING THROUGH LOUISIANA STATE UNIVERSITY HEALTH SCIENCES CENTER IN SHREVEPORT** [US/US]; 1501 Kings Highway, Shreveport, LA 71130 (US). **UNIVERSITY OF ALABAMA RESEARCH FOUNDATION** [US/US]; AB 1120G, 1530 3rd Avenue South, Birmingham, AL 35294-0111 (US). **LOMA LINDA UNIVERSITY** [US/US]; Anderson Street and Barton Road, Loma Linda, CA 92350 (US). **WAKE FOREST UNIVERSITY** [US/US]; Department of Physics, 208 Olin Physical Laboratory, Box 7507, 1834 Wake Forest University, Winston-Salem, NC 27109-7507 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GLADWIN, Mark, T.** [US/US]; 4927 Nebraska, Avenue, Northwest, Washington, DC 20008 (US). **SCHECHTER, Alan, N.** [US/US]; 5405 Beech Avenue, Bethesda, MD 20814 (US). **LEFER, David, J.** [US/US]; 5035 Beechwood Hills Drive, Shreveport, LA 71107 (US). **PATEL, Rakesh, P.** [GB/US]; 4172 River Walk Lane, Birmingham, LA 35294 (US). **HUNTER, Christian, J.** [US/US]; National Institutes of Health, Critical Care/NIDDK, 10 Center Drive, Building 10, Room 5D45, Bethesda, MD 20892 (US). **POWER, Gordon, G.** [US/US]; 1431 Henrietta Street, Redlands, CA 92373 (US). **KIM-SHAPIRO, Daniel, B.** [US/US]; 2065 Craig Street, Winston-Salem, NC 27103 (US). **PLUTA, Ryszard** [US/US]; National Institutes of Health NINDS, Building 10, Room 5D37, 10 Center Drive, MSC 1414, Bethesda, MD 20892-1414 (US). **OLDFIELD, Edward, H.** [US/US]; P.O. Box 309, Philomont, VA 22131 (US).

(74) Agent: **HARDING, Tanya, M.**; Klarquist, Sparkman, LLP, Suite 1600, One World Trade Center, 121 SW Salmon Street, Portland, OR 97204 (US).

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(54) Title: TREATMENT OF SPECIFIC CARDIOVASCULAR CONDITIONS WITH NITRITE

(57) Abstract: It has been surprisingly discovered that administration of nitrite to subjects causes a reduction in blood pressure and an increase in blood flow to tissues. The effect is particularly beneficial, for example, to tissues in regions of low oxygen tension. This discovery provides useful treatments to regulate a subject's blood pressure and blood flow, for example, by the administration of nitrite salts. Provided herein are methods of administering a pharmaceutically-acceptable nitrite salt to a subject, for treating, preventing or ameliorating a condition selected from : (a) ischemia-reperfusion injury (e.g., hepatic or cardiac or brain ischemia-reperfusion injury); (b) pulmonary hypertension (e.g., neonatal pulmonary hypertension); or (c) cerebral artery vasospasm.

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TREATMENT OF SPECIFIC CARDIOVASCULAR CONDITIONS WITH NITRITE

Cross Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/485,959, filed July 9, 2003, and No. 60/511,244, filed October 14, 2003, both of which are incorporated herein by reference in their entirety.

Government Interest Statement

Aspects of this invention were developed with government support under Grant Nos. HL58091 (D.B.K.-S.), and HL70146 (R.P.P.), both awarded by the National Institutes of Health. The government has certain rights in aspects of the invention. The government also may have certain rights in the invention due to at least one inventor's employment by the National Institutes of Health.

Background of the Disclosure

The last decade has seen an increase in the understanding of the critical role nitric oxide as a blood vessel dilator contributing to the regulation of blood flow and cardiovascular homeostasis. Nitric oxide may be oxidized in blood to nitrite (NO_2-), an anion considered to be an inert metabolic end product of such nitric oxide oxidation. *In vivo* plasma levels of nitrite have been reported to range from 150 to 1000 nM, and the nitrite concentration in aortic ring tissue has been reported to be in excess of 10,000 nM (Rodriguez *et al.*, *Proc Natl Acad Sci U S A, 100*, 336-41, 2003; Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; and Rassaf *et al.*, *Nat Med, 9*, 481-3, 2003). This potential storage pool for NO is in excess of plasma S-nitrosothiols, which have been reported to be less than 10 nM in human plasma (Rassaf *et al.*, *Nat Med, 9*, 481-3, 2003; Rassaf *et al.*, *Free Radic Biol Med, 33*, 1590-6, 2002; Rassaf *et al.*, *J Clin Invest, 109*, 1241-8, 2002; and Schechter *et al.*, *J Clin Invest, 109*, 1149-51, 2002). Mechanisms have been proposed for the *in vivo* conversion of nitrite to NO, for example, by enzymatic reduction by xanthine oxidoreductase or by non-enzymatic disproportionation/acidic reduction (Millar *et al.*, *Biochem Soc Trans, 25*, 528S, 1997; Millar *et al.*, *FEBS Lett, 427*, 225-8, 1998; Godber *et al.*, *J Biol Chem, 275*, 7757-63, 2000; Zhang *et al.*, *Biochem Biophys Res Commun, 249*, 767-72, 1998 [published erratum appears in *Biochem Biophys Res Commun 251*, 667, 1998]; Li *et al.*, *J Biol Chem, 276*, 24482-9, 2001; Li *et al.*, *Biochemistry, 42*, 1150-9, 2003; Zweier *et al.*, *Nat Med, 1*, 804-9, 1995; Zweier *et al.*, *Biochim Biophys Acta, 1411*, 250-62, 1999; and Samouilov *et al.*, *Arch Biochem Biophys, 357*:1-7, 1998).

Arterial-to-venous gradients of nitrite across the human forearm at rest and during regional NO synthase inhibition have been observed, with increased consumption of nitrite occurring with exercise (Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; Gladwin *et al.*, *Proc Natl Acad Sci USA, 97*, 11482-11487, 2000; and Cicinelli *et al.*, *Clin Physiol, 19*:440-2, 1999). Kelm and colleagues have reported that large artery-to-vein gradients of nitrite form across the human forearm during NO synthase inhibition (Lauer *et al.*, *Proc Natl Acad Sci USA, 98*, 12814-9, 2001). Unlike the more simple case of oxygen extraction across a vascular bed, nitrite may be both consumed, as

evidenced by artery-to-vein gradients during NO synthase inhibition and exercise, and produced in the vascular bed by endothelial nitric oxide synthase-derived NO reactions with oxygen.

At high concentrations, nitrite has been reported to be a vasodilator *in vitro* (Ignarro *et al.*, *Biochim Biophys Acta, 631*, 221-31, 1980; Ignarro *et al.*, *J Pharmacol Exp Ther, 218*, 739-49, 1981; Moulds *et al.*, *Br J Clin Pharmacol, 11*, 57-61, 1981; Gruetter *et al.*, *J Pharmacol Exp Ther, 219*, 181-6, 1981; Matsunaga *et al.*, *J Pharmacol Exp Ther, 248*, 687-95, 1989; and Laustiola *et al.*, *Pharmacol Toxicol, 68*, 60-3, 1991). The levels of nitrite shown to vasodilate *in vitro* have always been in excess of 100,000 nM (100 μM) and usually at millimolar concentrations.

Consistent with the high concentrations of nitrite required to vasodilate *in vitro*, when Lauer and colleagues infused nitrite into the forearm circulation of human subjects, they reported no vasodilatory effects, even with concentrations of 200 μM in the forearm (Lauer *et al.*, *Proc Natl Acad Sci USA, 98*, 12814-9, 2001). Lauer *et al.* reported that a "complete lack of vasodilator activity of intraartierial infusions of nitrite clearly rules out any role for this metabolite in NO delivery" and concluded that "physiological levels of nitrite are vasodilator-inactive." Furthermore, Rassaf and colleagues also failed to find a vasodilatory effect in humans following infusion of nitrite (Rassaf *et al.*, *J Clin Invest, 109*, 1241-8, 2002). Thus, *in vivo* studies have concluded that physiological levels of nitrites do not serve as a source for NO, and that physiological levels of nitrites do not have a role in regulating blood pressure.

Historically, nitrite has been used as a treatment for cyanide poisoning. High concentrations are infused into a subject suffering cyanide poisoning in order to oxidize hemoglobin to methemoglobin, which will bind cyanide. These high concentrations of nitrite produce clinically significant methemoglobinemia, potentially decreasing oxygen delivery. While these high concentrations of nitrite have been shown to decrease blood pressure in humans, the amount of methemoglobin formed precluded a use for nitrite in the treatment of other medical conditions.

Therefore, the state of the art was that nitrite was not a significant vasodilator at concentrations below 100 μM *in vitro*, and even when infused into humans at concentrations of 200 μM in the forearm. It was also the state of the art that nitrite was not converted to nitric oxide in the human blood stream.

Summary of the Disclosure

It has been surprisingly discovered that administration of pharmaceutically-acceptable salts of nitrite is useful in the regulation of the cardiovascular system. It has also been surprisingly discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. These effects surprisingly occur at doses that do not produce clinically significant methemoglobinemia. These discoveries now enable methods to prevent and treat conditions associated with the cardiovascular system, for example, high blood pressure, pulmonary hypertension, cerebral vasospasm and tissue ischemia-reperfusion injury. These discoveries also provide methods to increase blood flow to tissues, for example, to tissues in regions of low oxygen tension. It is particularly surprising that the nitrite does not need to be applied in an acidified

condition in order for it to be effective in regulating the cardiovascular system, and more particularly to act as a vasodilator *in vivo*.

It has now been surprisingly discovered by the inventors that nitrite can serve as a vasodilator in humans at much lower concentrations (as low as 0.9 μM) than have been used in the past for cyanide poisoning. The mechanism is believed to involve a reaction of nitrite with deoxygenated hemoglobin and red blood cells, to produce the vasodilating gas nitric oxide. This potent biological effect is observed at doses of nitrite that do not produce clinically significant methemoglobininemia (for instance, less than 20%, more preferably less than 5% methemoglobin in the subject).

It has been discovered that nitrite is converted to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. Further, it has been surprisingly discovered that administration of nitrite, for instance a pharmaceutically-acceptable salt of nitrite, to a subject causes a reduction in blood pressure and an increase in blood flow to tissues, for example, to tissues in regions of low oxygen tension. These discoveries now enable useful methods to regulate the cardiovascular system, for instance to prevent and treat malconditions associated with the cardiovascular system, for example, high blood pressure, or organs, tissues, or systems suffering a lack of or inadequate blood flow. Non-limiting examples of contemplated malconditions include stroke, heart disease, kidney disease and failure, eye damage including hypertensive retinopathy, diabetes, and migraines.

In one example embodiment, the present disclosure provides a method for decreasing a subject's blood pressure or increasing blood flow, including in a particular embodiment administering to the subject sodium nitrite at about 36 μmoles per minute into the forearm brachial artery.

The present disclosure additionally provides a method for increasing blood flow to a tissue of a subject, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite, such as a salt thereof, so as to increase blood flow to a tissue of the subject. The blood flow may be specifically increased in tissues in regions of low oxygen tension. The present disclosure also provides a method for decreasing a subject's blood pressure, comprising administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to decrease the subject's blood pressure.

The present disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure.

Also provided is a method for treating a subject having a hemolytic condition, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this

includes treating a subject having neonatal pulmonary hypertension. In some embodiments, this includes treating a subject having primary and/or secondary pulmonary hypertension. In some embodiments for treating subjects having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

Also contemplated herein are methods for treating, ameliorating, or preventing other conditions of or associated with blood flow, including vasospasm, stroke, angina, revascularization of coronary arteries and other arteries (peripheral vascular disease), transplantation (*e.g.*, of kidney, heart, lung, or liver), treatment of low blood pressure (such as that seen in shock or trauma, surgery and cardiopulmonary arrest) to prevent reperfusion injury to vital organs, cutaneous ulcers (*e.g.*, with topical, non-acidified nitrite salt), Raynauds phenomenon, treatment of hemolytic conditions (such as sickle cell, malaria, TTP, and HUS), hemolysis caused by immune incompatibility before and after birth, and other conditions listed herein.

Also provided herein are methods of administering a pharmaceutically-acceptable nitrite salt to a subject, for treating, preventing or ameliorating a condition selected from: (a) ischemia-reperfusion injury (*e.g.*, hepatic or cardiac or brain ischemia-reperfusion injury); (b) pulmonary hypertension (*e.g.*, neonatal pulmonary hypertension); or (c) cerebral artery vasospasm. Also contemplated are methods for treatment, prevention, and/or amelioration of gestational or fetal cardiovascular malconditions.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

Brief Description of the Figures

Figure 1 is a graph, depicting hemodynamic and metabolic measurements at baseline and during exercise in 18 subjects. **Figure 1A** shows effects on each of the indicated values without inhibition of NO synthesis. **Figure 1B** shows effects with inhibition of NO synthesis. *Key*: MAP – mean arterial pressure, mmHg; FBF – forearm blood flow, mL/min/100mL; O_2 saturation, %; pO_2 – venous oxyhemoglobin saturation, partial pressure of oxygen, mmHg; pH, units; * = $p<0.05$ vs. Baseline 1 or 2, respectively; ** = $p<0.01$ vs. Baseline 1 or 2, respectively; † = $p<0.05$ vs. Baseline 1; †† = $p<0.01$ vs. Initial Exercise.

Figure 2 is a graph, depicting effects of infusion of sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 18 healthy subjects. **Figure 2A** shows effects on each of the indicated values without inhibition of NO synthesis. **Figure 2B** shows effects with inhibition of NO synthesis. *Key as for Figure 1, plus*: Nitrite – venous nitrite, μM; NO-heme – venous iron-nitrosyl-hemoglobin, μM; and MetHb – venous methemoglobin, %; + = $p<0.01$ vs. Initial Exercise.

Figure 3 is a series of graphs, illustrating the effects of infusion of low-dose sodium nitrite into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. **Figure 3A** shows forearm blood flow at baseline and following a five-

minute infusion of $NaNO_2$. **Figure 3B** shows forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. **Figure 3C** shows venous levels of nitrite from the forearm circulation at the time of blood flow measurements. **Figure 3D** shows venous levels of S-nitroso-hemoglobin (S-NO) and iron-nitrosyl-hemoglobin (Hb-NO) at baseline and following nitrite infusion during exercise stress.

Figure 4 is a pair of graphs, showing formation of NO-hemoglobin adducts. **Figure 4A** shows formation of iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin, comparing baseline, with nitrite infusion, and nitrite infusion with exercise. **Figure 4B** compares formation of NO-hemoglobin adducts with hemoglobin-oxygen saturation in the human circulation, during nitrite infusion.

Figure 5A shows NO release following nitrite injections into solutions of PBS ("PBS"), deoxygenated red blood cells ("deoxy-RBC"), and oxygenated red blood cells ("oxy-RBC"). **Figure 5B** shows the rate of NO formation from nitrite mixed with PBS (first bar in each set), and oxygenated and deoxygenated red blood cells (second and third bar in each set, respectively).

Figure 6 is a mutlipanel figure showing nitrite therapy in hepatic ischemia-reperfusion injury. **Figure 6A** illustrates the experimental protocol used for murine model of hepatic ischemia-reperfusion injury. **Figure 6B** is a graph showing serum AST levels in mice following hepatic ischemia-reperfusion. *$p < 0.05$ vs. vehicle (0 μM) and **$p < 0.01$ vs. vehicle (0 μM) **Figure 6C** is a graph showing serum ALT levels in mice following hepatic ischemia-reperfusion. *$p < 0.05$ vs. vehicle (0 μM) and **$p < 0.01$ vs. vehicle (0 μM) **Figure 6D** is a representative photomicrographs of hepatic histopathology following 45 minutes of ischemia and 24 hours of reperfusion. **Figure 6E** is a bar graph showing pathological scoring of hepatic tissue samples following 45 minutes of ischemia and 24 hours of reperfusion. **Figure 6F** is a bar graph showing hepatocellular apoptosis as measured by TUNEL staining following 45 minutes of ischemia and 24 hours of reperfusion. ** $p < 0.001$ vs. I/R alone group

Figure 7 is a multipanel figure showing nitrite therapy in myocardial ischemia-reperfusion injury. **Figure 7A** illustrates the experimental protocol used for myocardial ischemia-reperfusion studies in mice. **Figure 7B** is a representative photomicrographs of the murine hearts following 30 minutes of myocardial ischemia and reperfusion. **Figure 7C** is a bar graph comparing myocardial area-at-risk (AAR) per left ventricle (LV), infarct size (INF) per AAR, and infarct per left ventricle in mice treated with nitrate or nitrite. **Figure 7D** is a bar graph comparing myocardial ejection fraction at baseline and following 45 minutes of myocardial ischemia and 48 hours of reperfusion. **Figure 7E** is a bar graph comparing left ventricular fractional shortening at baseline and following 45 minutes of myocardial ischemia and 48 hours of reperfusion.

Figure 8 is a series of graphs, illustrating blood and liver tissue levels of nitrite, RSNO and RxNO. **Figure 8A** shows blood nitrite, RSNO, and RxNO levels (μmol/L) in animals (n=3-5 per group) subjected to sham hepatic ischemia-reperfusion (I/R) or hepatic ischemia and either 1 or 30 minutes of reperfusion. ** $p < 0.001$ vs. sham **Figure 8B** shows liver tissue nitrite levels in mice (n=3-5 per group) subjected to hepatic ischemia-reperfusion (I/R) injury. **Figure 8C** shows liver tissue RSNO levels (μmol/L) in mice (n = 3-5 per group) subjected to hepatic ischemia and varying

periods of reperfusion. **Figure 8D** shows hepatic tissue RxNO levels (μmol/L) following hepatic ischemia and reperfusion in mice (n = 3-5 per group).

Figure 9 is a multipanel figure, illustrating nitrite mediated hepatoprotection and the nitric oxide and heme oxygenase-1 signaling pathways. **Figure 9A** is a graph, comparing serum aspartate aminotransferase (AST) levels in mice receiving saline vehicle, nitrite (24 μM), the nitric oxide (NO) scavenger PTIO, or nitrite (24 μM) + PTIO. **p < 0.01 vs. the vehicle group. **Figure 9B** is a graph comparing serum levels of AST in eNOS deficient (-/-) mice receiving saline vehicle or sodium nitrite (24 μM). **Figure 9C** is an image showing hepatic protein levels of heme oxygenase-1 (HO-1) determined using western blot analysis in sham operated animals and in animals subjected to hepatic ischemia (45 minutes) and reperfusion (5 hours). **Figure 9D** is a graph comparing serum AST levels in mice treated with nitrite (24 μM) or the HO-1 inhibitor zinc deuteroporphyrin bis glycol (ZnDPBG) in the setting of hepatic ischemia reperfusion injury.

Figure 10 is a series of panels, showing the effects of nitrite anion inhalation in newborn hypoxic lambs (*n*=7) **(Figure 10A)** on hemodynamic and metabolic measurements. After a hypoxic gas mixture (FiO_2 =0.12) had been started at time 0, nitrite by aerosol reduced pulmonary artery pressure (PAP) from hypoxic levels by 63 +/- 3% (*P* <0.01 versus hypoxic baseline) with little change in mean arterial pressure (MAP), cardiac output, or methemoglobin levels, but a marked increase in exhaled NO (*P* <0.01 compared to baseline). **Figure 10B** illustrates the effect of saline inhalation on pulmonary artery pressure in hypoxic lambs (*n*=7). **Figure 10C** is a multipanel graph, showing maximal effects of nitrite nebulization as compared to saline nebulization on PAP, MAP, and exhaled NO (eNO). Data are mean ± SEM.

Figure 11 illustrates effects of nitrite anion inhalation in newborn lambs during stable, normoxic (SaO_2 ~ 99 %) pulmonary hypertension induced by the infusion of an endoperoxide analog of thromboxane (U46619) (*n*=6). After infusion of U46619 was started at time 0, nitrite by aerosol reduced pulmonary artery pressure (PAP) from infusion baseline level by 23 ± 6% (*P* <0.05 compared to infusion baseline) with no measurable change in mean arterial pressure (MAP) and with a moderate increase in exhaled NO (*P* <0.01 compared to baseline).

Figure 12A compares the change in pulmonary arterial pressure (PAP), exhaled NO, and iron-nitrosyl-hemoglobin as measured by both chemiluminescence and electron paramagnetic resonance (EPR) after nitrite inhalation in animals with pulmonary hypertension induced with either hypoxia or infusion of the thromboxane analog U46199. Data for iron-nitrosyl-hemoglobin, measured by areas of output peaks after tri-iodide based reductive chemiluminescence **(Figure 12B)** and by depth of peak at 3350 Gauss in electron paramagnetic resonance (EPR) **(Figure 12C**; red line: drug induced, blue line: hypoxic) measured 20 minutes after nitrite inhalation was begun. **Figure 12D** shows change in mean pulmonary artery pressure during hypoxia after inhalation of nebulized sodium nitrite was related to blood pH, with increased vasodilation associated with decreasing pH (*r* = 0.57 *P* = 0.055). Data are mean ± SEM.

Figure 13 is a multipanel figure, showing duration of effect of NO gas inhalation (*n*=7) **(Figure 13A)** or nitrite nebulization (*n*=7) **(Figure 13B)** on hemodynamic and metabolic

measurements during hypoxic-induced pulmonary hypertension. Treatment with nitrite aerosol resulted in a rapid sustained reduction in hypoxic-induced pulmonary vasoconstriction and a graded increase in exhaled NO gas concentration with no change in mean arterial blood pressure. These results are contrasted to the rapid return in pulmonary artery pressure to hypoxic baseline after termination of inhaled NO gas (**Figure 13A**). Methemoglobin (Met Hb) concentrations increased from 2.1 ± 0.1 % during baseline to 2.8 ± 0.2% after nitrite nebulization (P <0.05). Note that the exhaled nitric oxide concentrations in **Figure 13A** reach the limit of detection during administration of inhaled nitric oxide (20 ppm). **Figure 13C** shows the change in pulmonary artery pressure (PAP) after aerosolization of nebulized nitrite and during the remaining hour of hypoxia following the termination of nitrite nebulization. **Figure 13D** shows the arterial plasma nitrite concentrations during the course of the experiment. **Figure 13E** shows the relationship between pulmonary artery pressure and exhaled NO after nitrite nebulization during hypoxia. Data are mean ± SEM.

Figure 14 is a multi-column (panel) figure depicting experiment design, biochemical and clinical results in a series of non-human primates that received intravenous nitrite to examine its effects on the development of vasospasm of the cerebral arteries and resulting ischemia. Each of the three columns represents a separate experimental group (control, low nitrite, and high nitrite). This figure describes experimental design (upper row: arrows pointing down marking the events; small arrows pointing up in the middle column representing daily boluses of nitrite), biochemical results (linear graphs: red, nitrite levels in blood; blue, nitrite levels in CSF; green, levels of nitrosylated protein/albumin in CSF; the brown bar graph represents the methemoglobin levels in blood), and mean blood pressure (the last grey bar graph) in samples collected during the experiment.

Figure 15 presents characteristic cerebral arteriograms before SAH (Day 0 (preinfusion); **Figure 15A, 15C**) and on day 7 after SAH (**Figure 15B, 15D**) in two animals: one control treated with intravenous infusion of saline at 2 μl/min for 14 days (**Figure 15A, 15B**) and one treated with intravenous nitrite at 870 μmol/min for 14 days (**Figure 15C, 15D**). In **Figure 15B**, the arrows point to the right middle cerebral artery (R MCA) in spasm. R ICA, the right internal carotid artery, R ACA, the right anterior cerebral artery.

Figure 16 depicts degree of vasospasm of the right middle cerebral artery (R MCA) in each animal from all experimental groups (8 control, 3 low dose, and 3 high dose of nitrite). R MCA vasospasm was assessed as the area of the proximal 14-mm segment of the right MCA by three blinded examiners using a computerized image analysis system (NIH Image 6.21). Arteriographic vasospasm was quantified relative to each animal baseline arteriogram. The mean values for saline vs. nitrite groups are represented by the circles; bars represent standard deviations. Statistical significance p<0.001.

Detailed Description of the Disclosure

I. Abbreviations

ANOVA analysis of variance

carboxy-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt
DCV	delayed cerebral vasospasm
deoxy-RBC	deoxygenated red blood cells
eNOS	endothelial NO synthase
FiO_2	fractional concentration of inspired oxygen
FBF	forearm blood flow
iNO	inhaled nitric oxide
I/R	ischemia-reperfusion
LCA	main coronary artery
L-NMMA	L-NG-monomethyl-arginine
LV	left ventricle
NO	nitric oxide
NOS	nitric oxide synthase
MAP	mean arterial pressure
MetHb	methemoglobin
oxy-RBC	oxygenated red blood cells
PBS	phosphate buffered saline
pO_2 (or Po_2)	partial oxygen pressure
SAH	subarachnoid hemorrhage
S-NO	S-nitroso-hemoglobin

II. Terms

Unless otherwise noted, terms used herein should be accorded their standard definitions and conventional usage. For example, one of skill in the art can obtain definitions for the terms used herein in dictionaries and reference textbooks, for example: *Stedman's Medical Dictionary* (26th Ed., Williams and Wilkins, Editor M. Spraycar, 1995); *The New Oxford American Dictionary* (Oxford University Press, Eds E. Jewell and F. Abate, 2001); *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 3rd Ed., Cold Spring Harbor Laboratory Press, 2001); and *Hawley's Condensed Chemical Dictionary*, 11th Ed. (Eds. N. I. Sax and R. J. Lewis, Sr., Van Nostrand Reinhold, New York, New York, 1987); *Molecular Biology and Biotechnology: a Comprehensive Desk Reference* (VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8)).

In order to facilitate review of the various embodiments, the following explanations of specific terms are provided:

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals.

Cerebral ischemia or ischemic stroke: A condition that occurs when an artery to or in the brain is partially or completely blocked such that the oxygen demand of the tissue exceeds the oxygen supplied. Deprived of oxygen and other nutrients following an ischemic stroke, the brain suffers damage as a result of the stroke.

Ischemic stroke can be caused by several different kinds of diseases. The most common problem is narrowing of the arteries in the neck or head. This is most often caused by atherosclerosis, or gradual cholesterol deposition. If the arteries become too narrow, blood cells may collect in them and form blood clots (thrombi). These blood clots can block the artery where they are formed (thrombosis), or can dislodge and become trapped in arteries closer to the brain (embolism).

Another cause of stroke is blood clots in the heart, which can occur as a result of irregular heartbeat (for example, atrial fibrillation), heart attack, or abnormalities of the heart valves. While these are the most common causes of ischemic stroke, there are many other possible causes. Examples include use of street drugs, traumatic injury to the blood vessels of the neck, or disorders of blood clotting.

Ischemic stroke is by far the most common kind of stroke, accounting for about 80% of all strokes. Stroke can affect people of all ages, including children. Many people with ischemic strokes are older (60 or more years old), and the risk of stroke increases with older ages. At each age, stroke is more common in men than women, and it is more common among African-Americans than white Americans. Many people with stroke have other problems or conditions which put them at higher risk for stroke, such as high blood pressure (hypertension), heart disease, smoking, or diabetes.

Fetal: A term describing the time period in the latter part of pregnancy when organ systems are functional and blood flow patterns are established for central critical organs, such as the heart, brain and lungs.

Hypoxia: Deficiency in the amount of oxygen reaching body tissues.

Injectable composition: A pharmaceutically acceptable fluid composition comprising at least one active ingredient, for example, a salt of nitrite. The active ingredient is usually dissolved or suspended in a physiologically acceptable carrier, and the composition can additionally comprise minor amounts of one or more non-toxic auxiliary substances, such as emulsifying agents, preservatives, pH buffering agents and the like. Such injectable compositions that are useful for use with the compositions of this disclosure are conventional; appropriate formulations are well known in the art.

Ischemia: A vascular phenomenon in which a decrease in the blood supply to a bodily organ, tissue, or part is caused, for instance, by constriction or obstruction of one or more blood vessels. Ischemia sometimes results from vasoconstriction or thrombosis or embolism. Ischemia can lead to direct ischemic injury, tissue damage due to cell death caused by reduced oxygen supply.

Ischemia/reperfusion injury: In addition to the immediate injury that occurs during deprivation of blood flow, ischemic/reperfusion injury involves tissue injury that occurs after blood flow is restored. Current understanding is that much of this injury is caused by chemical products and free radicals released into the ischemic tissues.

When a tissue is subjected to ischemia, a sequence of chemical events is initiated that may ultimately lead to cellular dysfunction and necrosis. If ischemia is ended by the restoration of blood flow, a second series of injurious events ensue producing additional injury. Thus, whenever there is a transient decrease or interruption of blood flow in a subject, the resultant injury involves two components - the direct injury occurring during the ischemic interval and the indirect or reperfusion injury that follows. When there is a long duration of ischemia, the direct ischemic damage, resulting from hypoxia, is predominant. For relatively short duration ischemia, the indirect or reperfusion mediated damage becomes increasingly important. In some instances, the injury produced by

reperfusion can be more severe than the injury induced by ischemia *per se*. This pattern of relative contribution of injury from direct and indirect mechanisms has been shown to occur in all organs.

Methemoglobin: The oxidized form of hemoglobin in which the iron in the heme component has been oxidized from the ferrous (+2) to the ferric (+3) state. This renders the hemoglobin molecule incapable of effectively transporting and releasing oxygen to the tissues. Normally, there is about 1% of total hemoglobin in the methemoglobin form.

Methemoglobinemia: A condition in which a substantial portion of the hemoglobin in the blood of a subject is in the form of methemoglobin, making it unable to carry oxygen effectively to the tissues. Methemoglobinemia can be an inherited disorder, but it also can be acquired through exposure to chemicals such as nitrates (nitrate-contaminated water), aniline dyes, and potassium chlorate. It is not the presence of methemoglobin but the amount that is important in the clinical setting. The following provides rough indications of symptoms associated with different levels of methemoglobin in the blood: < 1.7%, normal; 10-20%, mild cyanosis (substantially asymptomatic, though it can result in "chocolate brown" blood); 30-40%, headache, fatigue, tachycardia, weakness, dizziness; >35%, symptoms of hypoxia, such as dyspnea and lethargy; 50-60%, acidosis, arrhythmias, coma, convulsions, bradycardia, severe hypoxia, seizures; >70% usually results in death.

Neonate: A term describing the human or animal organism in the time period after birth and extending until the adjustments from fetal to newborn life are completed.

Nitrite: The inorganic anion $^-NO_2$ or a salt of nitrous acid (NO_2^-). Nitrites are often highly soluble, and can be oxidized to form nitrates or reduced to form nitric oxide or ammonia. Nitrite may form salts with alkali metals, such as sodium ($NaNO_2$, also known as nitrous acid sodium salt), potassium and lithium, with alkali earth metals, such as calcium, magnesium and barium, with organic bases, such as amine bases, for example, dicyclohexylamine, pyridine, arginine, lysine and the like. Other nitrite salts may be formed from a variety of organic and inorganic bases. In particular embodiments, the nitrite is a salt of an anionic nitrite delivered with a cation, which cation is selected from sodium, potassium, and arginine. Many nitrite salts are commercially available, and/or readily produced using conventional techniques.

Parenteral: Administered outside of the intestine, for example, not via the alimentary tract. Generally, parenteral formulations are those that will be administered through any possible mode except ingestion. This term especially refers to injections, whether administered intravenously, intrathecally, intramuscularly, intraperitoneally, or subcutaneously, and various surface applications including intranasal, intradermal, and topical application, for instance.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the compounds herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced

salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Peripheral Vascular Disease (PVD): A condition in which the arteries that carry blood to the arms or legs become narrowed or occluded. This interferes with the normal flow of blood, sometimes causing pain but often causing no readily detectable symptoms at all.

The most common cause of PVD is atherosclerosis, a gradual process in which cholesterol and scar tissue build up, forming plaques that occlude the blood vessels. In some cases, PVD may be caused by blood clots that lodge in the arteries and restrict blood flow. PVD affects about one in 20 people over the age of 50, or 8 million people in the United States. More than half the people with PVD experience leg pain, numbness or other symptoms, but many people dismiss these signs as "a normal part of aging" and do not seek medical help. The most common symptom of PVD is painful cramping in the leg or hip, particularly when walking. This symptom, also known as "claudication," occurs when there is not enough blood flowing to the leg muscles during exercise, such that ischemia occurs. The pain typically goes away when the muscles are rested.

Other symptoms may include numbness, tingling or weakness in the leg. In severe cases, people with PVD may experience a burning or aching pain in an extremity such as the foot or toes while resting, or may develop a sore on the leg or foot that does not heal. People with PVD also may experience a cooling or color change in the skin of the legs or feet, or loss of hair on the legs. In extreme cases, untreated PVD can lead to gangrene, a serious condition that may require amputation of a leg, foot or toes. People with PVD are also at higher risk for heart disease and stroke.

A **"pharmaceutical agent"** or **"drug"** refers to a chemical compound or other composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

Placenta: A vascular organ that provides for metabolic exchange between mother and fetus in mammals. It delivers oxygen, water, and nutrients to the fetus from the mother's blood and secretes the hormones necessary for successful pregnancy. In addition, it carries wastes away from the fetus to be processed in the mother's body.

Preeclampsia: A disease of unknown cause in pregnant women, characterized by hypertension, abnormal blood vessels in the placenta, and protein in the urine. It often but not always occurs with gestational diabetes or in diabetics. Additional symptoms may include water retention, leading to swelling in the face, hands and feet, and greater weight gain. Also called toxemia. Preeclampsia can lead to eclampsia if not treated. The only known cure for preeclampsia is delivery of the child.

Preventing or treating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified nitrite salt preparation is one in which the specified nitrite salt is more enriched than it is in its generative environment, for instance within a biochemical reaction chamber. Preferably, a preparation of a specified nitrite salt is purified such that the salt represents at least 50% of the total nitrite content of the preparation. In some embodiments, a purified preparation contains at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or more of the specified compound, such as a particular nitrite salt.

Reperfusion: Restoration of blood supply to tissue that is ischemic, due to decrease in blood supply. Reperfusion is a procedure for treating infarction or other ischemia, by enabling viable ischemic tissue to recover, thus limiting further necrosis. However, it is thought that reperfusion can itself further damage the ischemic tissue, causing reperfusion injury.

Subject: Living multi-cellular organisms, including vertebrate organisms, a category that includes both human and non-human mammals.

Therapeutic: A generic term that includes both diagnosis and treatment.

Therapeutically effective amount of [a vasodilator]: A quantity of compound, such as a nitrite salt, sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to treat or ameliorate relatively high blood pressure, or to measurably decrease blood pressure over a period of time, or to measurably inhibit an increase in blood pressure, in a subject.

An effective amount of a vasodilator may be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount will be dependent on the compound applied, the subject being treated, the severity and type of the affliction, and the manner of administration of the compound. For example, a therapeutically effective amount of an active ingredient can be measured as the concentration (moles per liter or molar-M) of the active ingredient (such as a pharmaceutically-acceptable salt of nitrite) in blood (*in vivo*) or a buffer (*in vitro*) that produces an effect.

By way of example, as described herein it is now shown that pharmaceutically-acceptable salts of nitrite (such as sodium nitrite) are effective as vasodilators at calculated dosages of about 0.6 to about 200 μM final concentration of nitrite in the circulating blood of a subject, which level can be determined empirically or through calculations. Specific levels can be reached, for instance, by providing less than about 200 mg or less nitrite in a single dose, or a dose provided over a period of time (*e.g.*, by infusion or inhalation). For instance, other dosages may be 150 mg, 100 mg, 75 mg, 50 mg or less. Specific example dosages of nitrite salts are provided herein, though the examples are not intended to be limiting. Exact dosage amounts will vary by the size of the subject being treated, the duration of the treatment, the mode of administration, and so forth.

Particularly beneficial therapeutically effective amounts of a vasodilator, such as a pharmaceutically-acceptable nitrite salt (*e.g.*, sodium nitrite), are those that are effective for vasodilation or increasing blood flow, but not so high that a significant or toxic level of methemoglobin is produced in the subject to which the vasodilator is administered. In specific embodiments, for instance, no more than about 25% methemoglobin is produced in the subject. More preferably, no more than 20%, no more than 15%, no more than 10%, no more than 8% or less methemoglobin is produced, for instance as little as 5% or 3% or less, in response to treatment with the vasodilator.

The compounds discussed herein have equal application in medical and veterinary settings. Therefore, the general term "subject being treated" is understood to include all animals (for example, humans, apes, laboratory animals, companion animals, etc.) that are or may be suffering from an aberration in blood pressure, such as hypertension.

Vasoconstriction. The diminution of the caliber or cross-sectional area of a blood vessel, for instance constriction of arterioles leading to decreased blood flow to a body part. This can be caused by a specific **vasoconstrictor**, an agent (for instance a chemical or biochemical compound) that causes, directly or indirectly, constriction of blood vessels. Such an agent can also be referred to as a **vasohypertonic** agent, and is said to have **vasoconstrictive** activity. A representative category of vasoconstrictors is the **vasopressor** (from the term pressor, tending to increase blood pressure), which term is generally used to refer to an agent that stimulates contraction of the muscular tissue of the capillaries and arteries.

Vasoconstriction also can be due to vasospasm, inadequate vasodilatation, thickening of the vessel wall, or the accumulation of flow-restricting materials on the internal wall surfaces or within the wall itself. Vasoconstriction is a major presumptive or proven factor in aging and in various clinical conditions including progressive generalized atherogenesis, myocardial infarction, stroke, hypertension, glaucoma, macular degeneration, migraine, hypertension and diabetes mellitus, among others.

Vasodilation. A state of increased caliber of the blood vessels, or the act of dilation of a blood vessel, for instance dilation of arterioles leading to increased blood flow to a body part. This can be caused by a specific **vasodilator**, an agent (for instance, a chemical or biochemical compound) that causes, directly or indirectly, dilation of blood vessels. Such an agent can also be referred to as a **vasohypotonic** agent, and is said to have **vasodilative** activity.

Vasospasm: Another cause of stroke occurs secondary to spasm of blood vessels supplying the brain. This type of stroke typically follows a subarchnoid aneurismal hemorrhage with a delayed development of vasospasm within 2-3 weeks of the bleeding event. A similar type of stroke may complicate sickle cell disease.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates

otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Hence "comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

III. Overview of Several Embodiments

It has been surprisingly discovered that administration of pharmaceutically-acceptable salts of nitrite is useful in the regulation of the cardiovascular system. It has also been surprisingly discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. These effects surprisingly occur at doses that do not produce clinically significant methemoglobinemia. These discoveries now enable methods to prevent and treat conditions associated with the cardiovascular system, for example, high blood pressure, pulmonary hypertension, cerebral vasospasm and tissue ischemia-reperfusion injury. These discoveries also provide methods to increase blood flow to tissues, for example, to tissues in regions of low oxygen tension. It is particularly surprising that the nitrite does not need to be applied in an acidified condition in order for it to be effective in regulating the cardiovascular system, and more particularly to act as a vasodilator *in vivo*.

Accordingly, the present disclosure provides in one embodiment a method for decreasing a subject's blood pressure, including administering to the subject sodium nitrite at about 36 μmoles per minute or less into the forearm brachial artery or intravenously.

The present disclosure also provides a method for decreasing a subject's blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to decrease (or lower, or reduce) the subject's blood pressure. Another embodiment is a method for treating a subject having a condition associated with elevated blood pressure, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure. Also provided is a method for treating a subject having a hemolytic condition, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The present disclosure additionally provides a method for increasing blood flow to a tissue of a subject, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite so as to increase blood flow to a tissue of the subject. Also provided is a method

for producing an amount of NO in a subject effective the decrease the subject's blood pressure, including administering a pharmaceutically-acceptable nitrite to the subject.

The present disclosure further provides a pharmaceutical composition comprising an effective amount of a pharmaceutically-acceptable nitrite and a carrier.

In some embodiments, the vascular complication is one or more selected from the group consisting of pulmonary hypertension (including neonatal pulmonary hypertension, primary pulmonary hypertension, and secondary pulmonary hypertension), systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, an ischemic central nervous system event, and death.

In some embodiments, nitrite is administered to neonates to treat pulmonary hypertension.

In some embodiments, the hemolytic condition includes one or more selected from: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, cardiopulomonary bypass, and hemodialysis.

In some embodiments, the decreased blood flow to the tissue is caused directly or indirectly by at least one of the following conditions: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

In some embodiments, the tissue is an ischemic tissue. In some embodiments, the administration is parenteral, oral, bucal, rectal, *ex vivo*, or intraocular. In some embodiments, the administration is peritoneal, intravenous, intraarterial, subcutaneous, inhaled, or intramuscular. In

some embodiments, the nitrite is administered to the subject in an environment of low oxygen tension, or acts in an area of the subject's body that displays relatively low oxygen tension. In some embodiments, the nitrite is administered as a pharmaceutically-acceptable salt of nitrite, such as, for instance, sodium nitrite, potassium nitrite, or arginine nitrite. In some embodiments, the nitrite is administered in combination with at least one additional active agent. It is specifically contemplated that, in certain embodiments, that the subject is a mammal, for instance, a human.

The disclosure further provides a method for treating a subject having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the subject an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this includes treating a subject having neonatal pulmonary hypertension. In some embodiments, this includes treating a subject having primary and/or secondary pulmonary hypertension. In some embodiments for treating subjects having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

The disclosure also provides suggestions for a means of treating hypertension and/or preeclampsia in pregnant women. Such therapy would include action of nitrites on spastic and diseased blood vessels within the placenta.

The disclosure also provides suggestions for treating, *in utero*, fetuses with cardiovascular anomalies, hypertension, and/or misdirected blood flow. In such approaches, nitrite may be administered by introduction into the amniotic cavity either directly or by osmotic minipumps, the latter to achieve sustained release throughout days and weeks of pregnancy.

Thus, there is provided herein a method for inducing vasodilation and/or increasing blood flow in a subject, which method involves administering to the subject an effective amount of a pharmaceutically-acceptable salt of nitrite for a sufficient period of time to induce vasodilation and/or increase blood flow in the subject. Non-limiting examples of pharmaceutically acceptable salts of nitrite include sodium nitrite, potassium nitrite, and arginine nitrite. In examples of the provided methods, the pharmaceutically-acceptable salt of nitrite reacts in the presence of hemoglobin in the subject to release nitric oxide.

It is a specific advantage of methods provided herein that the effective amount of the pharmaceutically-acceptable salt of nitrite administered to the subject does not induce toxic levels of methemoglobin, and in many embodiments does not induced formation of clinically significant amounts of methemoglobin in the subject. Therefore, contemplated herein are methods in which the effective amount of the pharmaceutically-acceptable salt of nitrite, when administered to the subject, induces production in the subject of no more than about 25% methemoglobin; no more than about 20% methemoglobin; no more than about 10% methemoglobin; no more than about 8% methemoglobin; or no more than about 5% methemoglobin. Beneficially, examples of the provided methods induce production of even less than 5% methemoglobin, for instance no more than about 3% methemoglobin, less than 3%, less than 2%, or even less than 1%.

In one specific example of a method for inducing vasodilation and/or increasing blood flow in a subject, sodium nitrite is administered by injection at about 36 μmoles per minute for at least five minutes into the forearm brachial artery of the subject.

The effective amount of the pharmaceutically-acceptable salt of nitrite is administered, in various embodiments, to a circulating concentration in the subject of about 0.6 to 240 μM, measured locally to the site of administration or generally in the subject. It is noted that the local level of nitrite is expected to be higher than the general circulating level particularly in short delivery regimens; in long term delivery regimens, such as delivery using a pump or injector, or by inhalation, the system-wide or general nitrite level is expected to near the level measured near the administration site.

Administration of the pharmaceutically-acceptable nitrite can be, for instance, parenteral, oral, bucal, rectal, *ex vivo*, or intraocular in certain embodiments. In various embodiments, it is also contemplated that the administration of the nitrite can be peritoneal, intravenous, intraarterial, subcutaneous, inhaled, intramuscular, or into a cardiopulmonary bypass circuit. Combinations of two or more routes of administration are also contemplated.

In various embodiments of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject is a mammal. It is particularly contemplated that the subject can be a human.

Combination therapy methods are contemplated, wherein the nitrite is administered in combination with at least one additional agent. By way of non-limiting examples, the additional agent is one or more selected from the list consisting of penicillin, hydroxyurea, butyrate, clotrimazole, arginine, or a phosphodiesterase inhibitor (such as sildenafil).

In another embodiment of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject has elevated blood pressure, and the method is a method for treating at least one vascular complication associated with the elevated blood pressure, or the subject has a hemolytic condition, and the method is a method for treating at least one vascular complication associated with the hemolytic condition. Optionally, the subject may have both elevated blood pressure and a hemolytic condition.

In examples of the methods provided herein, the at least one vascular complication is one or more selected from the group consisting of pulmonary hypertension, systemic hypertension, peripheral vascular disease, trauma, cardiac arrest, general surgery, organ transplantation, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, angina, an ischemia-reperfusion event, an ischemic central nervous system event, and death.

In examples of the methods in which the subject has a hemolytic condition, the hemolytic condition is one or more selected from the group consisting of sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia,

secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, and hemodialysis.

In yet another embodiment of the method for inducing vasodilation and/or increasing blood flow in a subject, the subject has a condition associated with decreased blood flow to a tissue, and the method is a method to increase blood flow to the tissue of the subject. For instance, in examples of this method, the decreased blood flow to the tissue is caused directly or indirectly by at least one condition selected from the group consisting of: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, rhabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

It is specifically contemplated in examples of this method that the tissue is an ischemic tissue, for instance one or more tissues selected from the group consisting of neuronal tissue, bowel tissue, intestinal tissue, limb tissue, lung tissue, central nervous tissue, or cardiac tissue.

Also provided are methods for inducing vasodilation and/or increasing blood flow in a subject having elevated blood pressure, wherein the elevated blood pressure comprises elevated blood pressure in the lungs. By way of example, it is contemplated that such subject in some instances has neonatal pulmonary hypertension, or primary and/or secondary pulmonary hypertension.

In examples of embodiments where the elevated blood pressure, or need for increased blood flow, in the subject comprises elevated blood pressure or need for increased blood flow in the lungs, the pharmaceutically-acceptable salt of nitrite is nebulized.

By way of example, in various embodiments the pharmaceutically-acceptable salt of nitrite is administered to a circulating concentration in the subject of no more than about 100 μM; no more than about 50 μM; no more than about 20 μM; no more than about 16 μM; or less than about 16 μM.

Another embodiment is a method for treating or ameliorating a condition selected from: (a) hepatic or cardiac or brain ischemia-reperfusion injury; (b) pulmonary hypertension; or (c) cerebral artery vasospasm, in a subject by decreasing blood pressure and/or increasing vasodilation in the subject, the method comprising administering sodium nitrite to the subject to decrease the blood pressure and/or increase vasodilation in the subject, thereby treating or ameliorating the condition.

In specific examples of this embodiment, the method is a method for treating or ameliorating hepatic or cardiac or brain ischemia-reperfusion injury. Optionally, the sodium nitrite is administered to the subject via injection, for instance, intravenous injection. In certain examples, the sodium nitrite is administered to a circulating concentration of about 0.6 to 240 μM.

In other specific examples of this embodiment, the method is a method for treating or ameliorating pulmonary hypertension, such as for instance neonatal pulmonary hypertension. Beneficially, in such methods the sodium nitrite can be administered to the subject by inhalation, for instance it can be nebulized. Optionally, in any of these methods, the sodium nitrite is administered at a rate of 270 μmol/minute, though other rates and circulating levels are contemplated.

Also provided in other examples of this embodiment are methods for treating or ameliorating cerebral artery vasospasm. Optionally, the sodium nitrite is administered to the subject via injection, for instance, intravenous injection. In examples of such methods, the sodium nitrite is administered at a rate of about 45 to 60 mg/kg.

In examples of the described methods, optionally the sodium nitrite can be administered in combination with at least one additional agent.

In any of the described methods, it is contemplated that the subject can be a mammal, such as for instance a human.

IV. Sodium Nitrite as an in vivo *vasodilator*

Nitrite anions are present in concentrations of about 150-1000 nM in the plasma and about 10 μM in aortic tissue. This represents the largest vascular storage pool of nitric oxide (NO), provided physiological mechanisms exist to reduce nitrite to NO. The vasodilator properties of nitrite in the human forearm and the mechanisms extant for its bioactivation have been investigated and results are reported herein. Sodium nitrite was infused at about 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers, resulting in a regional nitrite concentration of about 222 μM and an immediate about 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise, and resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Systemic concentrations of nitrite increased to about 16 μM and significantly reduced mean arterial blood pressure. In an additional six subjects, the dose of nitrite was reduced about 2-logs and infused at 360 nmoles per minute, resulting in a forearm nitrite concentration of about 2 μM and an about 22% increase in blood flow.

Nitrite infusions were associated with the formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin across the forearm vasculature. The

formation of NO-modified hemoglobin appears to result from the nitrite reductase activity of deoxyhemoglobin, linking tissue hypoxia and nitrite bioactivation.

These results indicate that physiological levels of blood and tissue nitrite represent a major bioavailable pool of NO that contributes to vaso-regulation and provides a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins. Substantial blood flow effects of nitrite infusion into the brachial artery of normal human subjects results from forearm nitrite concentrations as low as about 0.9μM.

By way of example, as described herein it is now shown that pharmaceutically-acceptable salts of nitrite (such as sodium nitrite) are effective as vasodilators at calculated dosages of about 0.6 to about 200 μM final concentration of nitrite in the circulating blood of a subject. Specific circulating levels (locally or generally in the subject) can be reached, for instance, by providing less than about 200 mg or less nitrite in a single dose, or a dose provided over a period of time (*e.g.*, by infusion or inhalation). For instance, other dosages may be 150 mg, 100 mg, 75 mg, 50 mg or less. Specific example dosages of nitrite salts are provided herein, though the examples are not intended to be limiting. Exact dosage amounts will vary by the size of the subject being treated, the duration of the treatment, the mode of administration, and so forth.

Infusion rates can be calculated, for any given desired target circulating concentration, by using the following equation:

Infusion rate (μM/min) = target concentration (μmol/L, or μM) x Clearance (L/min)
where Clearance (L/min) = 0.015922087 x weight of the subject (kg) ^0.8354

The rate of clearance has been calculated based on empirical results, including those reported herein.

By way of example, when sodium nitrite is infused into a human forearm at 36 micromoles (μMol) per minute, the concentration measured coming out of forearm is about 222 μM and about 16 μM in whole body, after 15 minutes infusion. The background level of circulating nitrite in mammals is low, around 150-500 nanoM.

Particularly beneficial therapeutically effective amounts of a vasodilator, such as a pharmaceutically-acceptable nitrite salt (*e.g.*, sodium nitrite), are those that are effective for vasodilation or increasing blood flow, but not so high that a significant or toxic level of methemoglobin is produced in the subject to which the vasodilator is administered. In specific embodiments, for instance, no more than about 25% methemoglobin is produced in the subject. More preferably, no more than 20%, no more than 15%, no more than 10%, no more than 8% or less methemoglobin is produced, for instance as little as 5% or 3% or less, in response to treatment with the vasodilator.

By way of specific example, nitrite can be infused at concentrations less than 40 μMol per minute intravenously or intraarterially, or given by mouth. Importantly, doses used are less than those used for the treatment of cyanide poisoning, which are designed to induce clinically significant methemoglobinemia. Surprisingly, the doses described herein for the treatment/prevention of

cardiovascular conditions produce significant and beneficial clinical effects without clinically significant methemoglobin production.

Relatively complex inorganic/organic nitrite compounds and nitrate compounds have been utilized clinically to treat disorders, including angina. These drugs (*e.g.*, glyceryl trinitrate) suffer from tolerance (requiring increases in dosage in order to maintain the same effect), however, and are distinct vasodilators compared to nitrite. For example, the former require cellular thiols for metabolism, whereas nitrite or the nitrite salts discussed herein (*e.g.*, sodium nitrite) do not.

V. A mechanism of iron-nitrosyl- and S-nitroso-hemoglobin formation in vivo

The levels of both iron-nitrosyl- and S-nitroso-hemoglobin formed *in vivo* in this study are striking. During a transit time of less than 10 seconds through the forearm circulation during exercise, infused nitrite (200 μM regional concentration) produced approximately 750 nM iron-nitrosyl-hemoglobin and 200 nM SNO-Hb. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation, which fell during exercise stress, measured from the antecubital vein by co-oximetry (for iron-nitrosyl-hemoglobin $r=-0.7$, $P<0.0001$; for S-nitroso-hemoglobin $r=-0.45$, $P=0.04$; Figure 4B). Addition of 200 μM nitrite to whole blood at different oxygen tensions (0-100%) recapitulated the *in vivo* data with increasing concentrations of iron-nitrosyl hemoglobin being formed at lower oxygen tensions (for iron-nitrosyl-hemoglobin $r=-0.968$, $P<0.0001$; for S-nitroso-hemoglobin $r=-0.45$, $P=0.07$), strongly suggesting that the NO and SNO formation was dependent on the reaction of nitrite with deoxyhemoglobin.

These data are consistent with the reaction of nitrite with deoxyhemoglobin to form NO and iron-nitrosyl-hemoglobin (Doyle *et al.*, *J Biol Chem, 256*, 12393-12398, 1981). Nitrite is first reduced to form NO and methemoglobin with a rate constant of 2.9 $M^{-1}sec^{-1}$ (measured at 25°C, pH 7.0). This reaction will be pseudo-first order, governed by the amounts (20 mM) of intra-erythrocytic hemoglobin, and limited by the rate of nitrite uptake by the erythrocyte membrane. NO then binds to deoxyhemoglobin to form iron-nitrosyl-hemoglobin, escapes the erythrocyte, or reacts with other higher oxides, such as NO_2, to form N_2O_3 and S-nitroso-hemoglobin.

Equation series 1

NO_2^- (nitrite) + $HbFe^{II}$ (deoxyhemoglobin) + $H^+ \rightarrow$ $HbFe^{III}$ (methemoglobin) + NO + OH^-

NO + $HbFe^{II}$ (deoxyhemoglobin) $\rightarrow$ $HbFe^{II}NO$ (iron-nitrosyl-hemoglobin)

The formation of significant amounts of S-nitroso-hemoglobin *in vivo* during nitrite infusion was also observed. Luschinger and colleagues (*Proc Natl Acad Sci USA, 100*, 461-6, 2003) recently proposed that nitrite reacts with deoxyhemoglobin to make iron-nitrosyl-hemoglobin, with subsequent "transfer" of the NO to the cysteine 93 to form S-nitroso-hemoglobin mediated by reoxygenation and quaternary T to R transition of hemoglobin. However, a direct transfer of NO from the heme to the thiol requires NO oxidation to NO+ and such "cycling" has not been reproduced by other research groups. Fernandez and colleagues have recently suggested that nitrite catalyzes the

reductive nitrosylation of methemoglobin by NO, a process that generates the intermediate nitrosating species dinitrogen teraoxide (N_2O_3) (*Inorg Chem, 42*, 2-4, 2003). However, nitrite reactions with hemoglobin provide ideal conditions for NO and S-nitrosothiol generation along the oxygen gradient as nitrite reacts with deoxyhemoglobin to form NO and with oxyhemoglobin to form nitrogen dioxide (NO_2) radical. NO_2 participates in radical-radical reactions ($k=10^9$ $M^{-1}sec^{-1}$) with NO to form N_2O_3 and S-nitrosothiol. Additional chemistry of nitrite with hemoglobin produces reactive oxygen metabolites (such as superoxide and hydrogen peroxide; Watanabe *et al.*, *Acta Med Okayama 35*, 173-8, 1981; Kosaka *et al.*, *Biochim Biophys Acta 702*, 237-41, 1982; and Kosaka *et al.*, *Environ Health Perspect 73*, 147-51, 1987). Chemistry involving such NO radical- oxygen radical reactions provides competitive pathways for S-nitrosothiol formation in the presence of high affinity NO sinks, such as hemoglobin.

VI. Physiological Considerations

The last decade has seen an increase in the understanding of the critical role nitric oxide (NO) plays in vascular homeostasis. The balance between production of NO and scavenging of NO determines NO bioavailability, and this balance is carefully maintained in normal physiology. The homeostatic, vasoregulatory system is apparently fine-tuned to scavenge excess NO to limit gross endocrine actions while allowing for sufficient local NO necessary for regional tonic vasodilation. However, rapid NO scavenging by cell-free hemoglobin disrupts this balance (Reiter *et al.*, *Nat Med* 8, 1383-1389, 2002). Under normal physiological conditions, hemoglobin is rapidly and effectively cleared by the hemoglobin scavenger system. However, chronic hemolytic conditions, such as sickle cell disease, result in the daily release of substantial quantities of hemoglobin into the vasculature, suggesting that cell-free hemoglobin may have major systemic effects on NO bioavailability. A current focus of research attempts to explain and treat the vascular complications common to many chronic hemolytic conditions, such as pulmonary hypertension, cutaneous ulceration and acute and chronic renal failure. Similarly, a number of clinical diseases and therapies such as acute hemolytic crises, hemolysis during cardiopulmonary bypass procedures, transfusion of aged blood, and myoglobinuria following muscle infarction are often complicated by acute pulmonary and systemic hypertension, acute renal failure, intravascular thrombosis, ischemic central nervous system events and/or death.

It is demonstrated herein that nitrite produces vasodilation in humans associated with nitrite reduction to NO by deoxyhemoglobin. Remarkably, systemic levels of 16 μM resulted in systemic vasodilation and decreased blood pressure, and regional forearm levels of only 1-2 μM significantly increased blood flow at rest and with exercise stress. Furthermore, conversion of nitrite to NO and S-nitrosothiol was mediated by reaction with deoxyhemoglobin, providing a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial/tissue heme proteins. While high concentrations of hemoglobin in red cells, coupled with the near diffusion-limited reaction rates (~10^7 $M^{-1}s^{-1}$) of NO with hemoglobin, seem to prohibit NO from being exported from the red blood cell, the data presented herein argue to the contrary. While not intending to be limiting, perhaps unique

characteristics of the erythrocyte membrane, with a submembrane protein and methemoglobin-rich microenvironment, and the relative lipophilic nature of NO, allow compartmentalized NO production at the red blood cell membrane. This, coupled with the small yields of NO necessary for vasodilation, could account for the export of NO despite these kinetic constraints. It is further proposed that *in vivo* chemistry for the conversion of nitrite to NO and S-nitrosothiol by reaction with deoxyhemoglobin and methemoglobin provides a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial tissue heme proteins.

Three factors uniquely position nitrite, rather than S-nitrosothiol, as the major vascular storage pool of NO: 1) Nitrite is present in substantial concentrations in plasma, erythrocytes and in tissues (Rodriguez *et al., Proc Natl Acad Sci USA* 100:336-341, 2003). 2) Nitrite is relatively stable, because it is not readily reduced by intracellular reductants, as are S-nitrosothiols (Gladwin *et al., J Biol Chem* 21:21, 2002) and its reaction rate with heme proteins is 10,000 times less than that of authentic NO. 3) Nitrite is only converted to NO by reaction with deoxyhemoglobin (or presumably deoxy-myoglobin, -cytoglobin, and -neuroglobin) and its "leaving group" is the met(ferric)heme protein which will not scavenge and inactivate NO (Doyle *et al., J Biol Chem* 256:12393-12398, 1981). Therefore, this pool provides the ideal substrate for NO generation during hypoxia, providing a novel mechanism for hypoxic vasodilation.

Because a deoxyhemoglobin-nitrite reductase system would result in NO formation in deoxygenating blood, such a system links hemoglobin oxygenation status to NO generation, the principle previously ascribed to S-nitroso-hemoglobin (Jia *et al., Nature* 380:221-226, 1996). Hemoglobin possesses anionic binding cavities that retain nitrite (Gladwin *et al., J Biol Chem* 21:21, 2002) and nitrite is taken up by erythrocytes through the anion exchange protein (AE1 or Band 3) or through the membrane as nitrous acid (a pH dependent process that accelerates nitrite uptake during tissue hypoxia (Shingles *et al., J Bioenerg Biomembr* 29:611-616, 1997; May *et al., Am I Physiol Cell Physiol* 279:C1946-1954, 2000). Such nitrite would provide a steady source of NO, NO_2 and S-nitrosothiol generation that would occur preferentially in hypoxic vascular territories. Because the AE1 protein binds both deoxyhemoglobin and methemoglobin and may channel nitrite, AE1 could serve to localize catalytic NO and S-nitrosothiol generation at the erythrocyte membrane, where the relatively lipophilic NO, NO_2 and N_2O_3 could react in the vicinal lipid bilayer (Figure 5). The erythrocyte membrane is lined by an unstirred outer diffusion barrier and an inner methemoglobin rich protein matrix that might further promote such NO and NO_2 chemistry (Coin *et al., J Biol Chem* 254:1178-1190, 1979; Liu *et al., J Biol Chem* 273:18709-18713, 1998; Han *et al., Proc Natl Acad Sci USA* 99:7763-7768, 2002).

This model is consistent with the *in vitro* observations of Pawloski and colleagues (Pawloski *et al., Nature* 409:622-626, 2001) showing that S-nitrosation of hemoglobin and AE1 occurs in the erythrocyte membrane after treatment of deoxygenated red blood cells with NO solutions (which contain significant-more than 50 μM- contaminating nitrite; Fernandez,*et al.* Inorg Chem 42:2-4, 2003). Further, N_2O_3 generated at the membrane could directly nitrosate the abundant intra-erythrocytic glutathione, eliminating the requirement of transnitrosation reactions with S-nitroso-

hemoglobin and thus facilitating rapid export of low molecular weight S-nitrosothiol by simple diffusion across the erythrocyte membrane (Figure 5). A nitrite-hemoglobin chemistry supports a role for the red cell in oxygen-dependent NO homeostasis and provides a mechanism for the observations of multiple research groups that red blood cells and plasma "loaded" with NO, by exposure to NO in high concentration in solution or to NO gas or donors (in equilibria with high concentrations of nitrite), can export NO and induce vasodilation *in vitro* and *in vivo* (Rassaf *et al., J Clin Invest* 109:1241-1248, 2002; Fox-Robichaud *et al., J Glitz Invest* 101:2497-2505, 1998; McMahon *et al., Nat Med* 3:3, 2002; Cannon *et al., J Clin Invest* 108:279-287, 2001; Gladwin *et al., J Biol Chem* 21:21, 2002; Gladwin *et al., Circulation* 107:271-278, 2003; Schechter *et al., N Engl J Med* 348:1483-1485, 2003).

In addition to the reaction of nitrite with deoxyhemoglobin, reactions with deoxy-myoglobin, -cytoglobin and -neuroglobin or with other endothelial cell heme proteins may also be important. Such chemistry would occur between tissue nitrite and deoxy-myoglobin in vascular and skeletal muscle, thus contributing to hypoxic vasodilation and hypoxic potentiation of NO donors. The P_{50} of these globin monomers is approximately 3-5 mm Hg, placing their equilibrium deoxygenation point in the range of tissue pO_2 (0-10 mm Hg) during metabolic stress, such as exercise. Such a low oxygen tension reduces oxygen availability as substrate for NO synthesis, however, the tissue nitrite stores could then be reduced to NO and 5-nitrosothiol, thus sustaining critical vasodilation.

VII. Methods of Use

Therapeutic application of nitrite now can be used to provide selective vasodilation in a subject, and particularly to hypoxemic and ischemic tissue in the subject, and will be useful to treat hemolytic conditions such as sickle cell disease, where free hemoglobin released during hemolysis scavenges NO and disrupts NO-dependent vascular function. Nitrite is expected to not only inhibit the ability of free hemoglobin to scavenge NO by oxidizing it to methemoglobin, but also to generate NO in tissue beds with low oxygen tension. Thus, the applied nitrite will preferentially release nitric oxide at areas of low oxygen tension, thereby providing localized vasodilation and/or increased blood flow.

Nitrites can be administered to a subject to increase blood flow to a tissue of the subject, for example, to increase blood flow to a tissue, for instance a tissue with low oxygen tension; to cause vasodilation; to decrease a subject's blood pressure; to treat a subject having a condition associated with elevated blood pressure; to treat a hemolytic condition; to treat vascular complications associated with treatments or conditions that cause hemolysis; to treat pulmonary hypertension, cerebral vasospasm, or low blood flow to organs (such as ischemia reperfusion injury to organs including brain, heart, kidney, placenta, and liver); and/or to treat organs before and after transplantation.

Nitrite has vasodilatory properties *in vivo*

The vasodilator properties of nitrite and the mechanisms for its bioactivation were investigated as described herein. Sodium nitrite infused at 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers resulted in a regional nitrite concentration of 222 μM and, surprisingly, a 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise. The nitrite infusion also surprisingly resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Increased systemic concentrations of nitrite (16 μM) significantly reduced mean arterial blood pressure.

In an additional ten subjects, the dose of nitrite was reduced 2-logs, resulting in a forearm nitrite concentration of 2 μM at rest and 0.9 μM during exercise (Figure 3). These concentrations of nitrite surprisingly significantly increased blood flow at rest and during NO synthase inhibition, with and without exercise.

Nitrite infusions were associated with the rapid formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin across the forearm vasculature. Formation of these NO-Hb adducts was inversely proportional to the oxyhemoglobin saturation. Additionally, vasodilation of rat aortic rings and the formation of both NO gas and NO-modified hemoglobin from the nitrite reductase activity of deoxyhemoglobin and deoxygenated erythrocytes was observed, a result that links tissue hypoxia, hemoglobin allostery, and nitrite bioactivation. These results indicate that physiological levels of blood and tissue nitrite are a major bioavailable pool of NO that contributes to vaso-regulation and provide a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins in tissue and/or the erythrocyte.

The findings described herein that administration of nitrite reduces blood pressure and increases blood flow are unexpected and surprising because published reports to date teach the person of ordinary skill in the art that pharmacological levels of nitrites (below about 100-200 μM), when administered to subjects, lack intrinsic vasodilatory properties (Lauer *et al.*, *Proc Natl Acad Sci USA,* 98:12814-9, 2001).

It is also believed that pharmaceutically acceptable salts of nitrite can be infused into patients with hemolytic disease, such as sickle cell disease, to improve blood flow, limit ischemia-reperfusion tissue injury, and oxidize cell-free plasma Hb. These effects should be useful in the treatment of sickle cell vaso-occlusive pain crisis, stroke (brain ischemia) and the acute chest syndrome.

Cytoprotective Effects of Nitrite during Ischemia-reperfusion of the Heart and Liver

The anion nitrite (NO_2^-) forms as a consequence of nitric oxide (NO) oxidation and is present at concentrations of 0.3-1.0 μM in plasma and 1-20 μM in tissue (Gladwin *et al.*, *Proc Natl Acad Sci U S A* 97:11482-11487, 2000; Rodriguez *et al.*, *Proc Natl Acad Sci U S A* 100:336-341, 2003; Rassaf *et al.*, *Nat Med* 9:481-483, 2003; Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Gladwin *et al.*, *J Clin Invest* 113:19-21, 2004). Nitrite has been historically considered an inert

metabolic end product with limited intrinsic biological activity (Lauer *et al.*, *Proc Natl Acad Sci U S A* 98:12814-12819, 2001; McMahon, *N Engl J Med* 349:402-405; author reply 402-405, 2003; Pawloski, *N Engl J Med* 349:402-405; author reply 402-405, 2003). Recent data from our group and others suggest that nitrite may be reduced to NO during hypoxia and acidosis (Gladwin *et al.*, *Proc Natl Acad Sci U S A* 97:11482-11487, 2000; Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*,. *J Biol Chem* 278:46349-46356, 2003; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004). At extremely low tissue pH and PO_2, nitrite may be reduced to NO by disproportionation (acidic reduction; Zweier *et al.*, *Nat Med* 1:804-809, 1995) or by the enzymatic action of xanthine oxidoreductase (Millar *et al.*, *FEBS Lett* 427:225-228, 1998; Zhang *et al.*, *Biochem Soc Trans* 25:524S, 1997; Godber *et al.*, *J Biol Chem* 275:7757-7763, 2000; Li *et al.*, *J Biol Chem* 276:24482-24489, 2001).

Nitrite represents a circulating and tissue storage form of nitric oxide (NO) whose bioactivation is mediated by the nitrite reductase activities of deoxyhemoglobin. Because the rate of NO generation from nitrite is linearly dependent on reductions in oxygen and pH, we hypothesized that nitrite would be reduced to NO in ischemic tissue and exert NO-dependent protective effects. Solutions of sodium nitrite were administered in the setting of hepatic and cardiac ischemia-reperfusion (I/R) injury in mice. In hepatic I/R, nitrite exerted profound dose dependent protective effects on cellular necrosis and apoptosis with highly significant protective effects observed at near-physiological nitrite concentrations (0.6 μM). In myocardial I/R injury, nitrite reduced cardiac infarct size by 67% and significantly improved post-ischemic left ventricular ejection fraction. Consistent with hypoxia dependent nitrite bioactivation, nitrite was reduced to NO, S-nitrosothiols, N-nitrosamines and iron-nitrosylated heme proteins within 1-30 minutes of reperfusion. Nitrite-mediated protection was dependent on NO generation and independent of eNOS and HO-1. These results suggest that nitrite is a biological storage reserve of NO subserving a critical function in tissue protection from ischemic injury. These studies evince an unexpected and novel therapy for diseases such as myocardial infarction, organ preservation and transplantation, and shock states.

Although reperfusion of ischemic tissues provides oxygen and metabolic substrates necessary for the recovery and survival of reversibly injured cells, reperfusion itself actually results in the acceleration of cellular necrosis (Braunwald *et al.*, *J. Clin. Invest.* 76:1713-1719, 1985). Ischemia-reperfusion is characterized by the formation of oxygen radicals upon reintroduction of molecular oxygen to ischemic tissues resulting in widespread lipid and protein oxidative modifications of cellular proteins, mitochondrial injury, and tissue apoptosis and necrosis (McCord *et al.*, *Adv Myocardiol* 5:183-189, 1985). In addition, following reperfusion of ischemic tissues blood flow may not return uniformly to all portions of the ischemic tissues, a phenomenon that has been termed the "no-reflow" phenomenon (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). Reductions in blood flow following reperfusion are thought to contribute to cellular injury and necrosis (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). The sudden re-introduction of blood into ischemic tissue also results in a dramatic increase in calcium delivery to the previously ischemic tissue (*i.e.*, "calcium paradox") resulting in massive tissue disruption, enzyme release, reductions in high energy phosphate

stores, mitochondrial injury, and necrosis (Nayler, *Amer. J. Path.* 102:262, 1981; Shen *et al.*, *Amer. J. Path* 67:417-440, 1972). Recent studies have also indicated that the ischemia-reperfusion injury is also characterized by an inappropriate inflammatory response in the microcirculation resulting in leukocyte-endothelial cell interactions that are mediated by the upregulation of both leukocyte and endothelial cell adhesion molecules (Lefer *et al.*, *Cardiovasc Res* 32:743-751, 1996; Entman *et al.*, *Faseb J* 5:2529-2537, 1991). Intensive research efforts have been focused on ameliorating various pathophysiological components of ischemia-reperfusion injury to limit the extent of tissue injury and necrosis.

NO, NO donors, and NO synthase activation or transgenic over-expression have been shown to exert protective effects on this process in a number of models (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993; Nakanishi *et al.*, *Am J Physiol* 263:H1650-1658, 1992; Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003; Kanno *et al.*, *Circulation* 101:2742-2748, 2000), but in other models appears harmful (Flogel *et al.*, *J Mol Cell Cardiol* 31:827-836. 1999; Menezes *et al.*, *Am J Physiol* 277:G144-151, 1999; Woolfson *et al.*, *Circulation* 91:1545-1551, 1995; Schulz, R. *et al.*, *Cardiovasc Res* 30:432-439, 1995). Evaluation of these studies suggests a critical effect of dose and duration of NO exposure, resulting in a narrow therapeutic safety window for NO in ischemia-reperfusion pathophysiology (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001; Wink *et al.*, *Am J Physiol Heart Circ Physiol* 285:H2264-2276, 2003). An additional limitation is that NO formation from NO synthase requires oxygen as substrate, a molecule whose availability becomes limited during ischemia.

We therefore considered the use of nitrite in this context for the following reasons: (1) It is a naturally occurring substance with no potentially toxic "leaving group" (2), it is selectively reduced to NO in tissues with low oxygen tension and low pH (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004; Doyle *et al.*, *J Biol Chem* 256:12393-12398, 1981; Luchsinger *et al.*, *Proc Natl Acad Sci US A* 100:461-466, 2003), (3) its activation does not require molecular oxygen (Cosby *et al.*, *Nat Med* 9:1498-1505, 2003), and (4) NO is known to maintain heme proteins in a reduced and liganded state (Herold *et al.*, *Free Radic Biol Med* 34:531-545, 2003; Herold *et al.*, *J Biol Inorg Chem* 6:543-555, 2001; Fernandez *et al.*, *Inorg Chem* 42:2-4, 2003), limit free iron and heme mediated oxidative chemistry (Kanner *et al.*, *Arch Biochem Biophys* 237:314-321, 1985; Kanner *et al.*, *Lipids* 20:625-628, 1985; Kanner *et al.*, *Lipids* 27:46-49, 1992), transiently inhibit cytochome c oxidase and mitochondrial respiration (Torres *et al.*, *FEBS Lett* 475:263-266, 2000; Brown *et al.*, *FEBS Lett* 356:295-298, 1994; Cleeter *et al.*, *FEBS Lett* 345:50-54, 1994; Rakhit *et al.*, *Circulation* 103:2617-2623, 2001), and modulate apoptotic effectors (Mannick *et al.*, *Science* 284:651-654, 1999), all mechanisms that might participate in cytotoxicity following severe ischemia.

Nitric oxide has been shown to quench oxygen free radicals in a transient ischemia and reperfusion injury animal models (Mason *et al.*, *J Neurosurg* 93: 99-107, 2000), significantly limiting

volume of stroke (Pluta *et al.*, *Neurosurgery*, 48:884-892, 2001). Therefore, nitrite via releasing NO in the area of reperfusion may also have the same beneficial effect on stroke via limiting oxygen free radicals presence after reperfusion.

Furthermore, the selective opening of blood-tumor barrier by NO facilitates penetration of chemotherapeutic agents into the brain tumor (Weyerbrock *et al.*, *J. Neurosurgery*, 99:728-737, 2003); it is believed that this will also enhance penetration of other agents, particularly therapeutic agents such as radiation therapy, brain cancer. Therefore, due to hypoxic conditions within the brain tumor it is possible that nitrite can also selectively open the blood-tumor barrier providing beneficial effect in combination with chemotherapy.

Inhaled Nebulized Nitrite is a Pulmonary Vasodilator

Persistent pulmonary hypertension of the newborn occurs with an incidence of 0.43–6.8/1,000 live births and is associated with mortality rates between 10–20% (Walsh-Sukys *et al.*, *Pediatrics* 105, 14-20, 2000). Survivors may develop neurodevelopmental and audiological impairment (46%), cognitive delays (30%), hearing loss (19%) and a high rate of rehospitalization (22%) (Lipkin *et al.*, *J Pediatr* 140, 306-10, 2002).

Pulmonary hypertension occurs as a primary or idiopathic disease (Runo & Loyd, *Lancet* 361:1533-44, 2003; Trembath & Harrison, *Pediatr Res* 53:883-8, 2003), as well as secondary to a number of systemic and pulmonary diseases (Rubin, *N Engl J Med* 336:111-7, 1997). Regardless of etiology, pulmonary hypertension is associated with substantial morbidity and mortality. Newborn infants and adults with pulmonary disease often develop systemic hypoxemia, reduced oxyhemoglobin saturation and increased pulmonary vascular resistance (Rubin, *N Engl J Med* 336:111-7, 1997; Haworth, *Heart* 88:658-64, 2002). Therapeutically administered inhaled nitric oxide (NO) decreases pulmonary vascular resistance in newborns and adults and improves ventilation-to-perfusion matching and oxygenation; in newborns, inhaled NO reduces chronic lung damage and reduces the need for extracorporeal membrane oxygenation. Randomized placebo-controlled trials of inhaled NO therapy for term and near-term newborns with severe hypoxic respiratory failure demonstrated an improvement in hypoxemia and reduced need for extracorporal membrane oxygenation (Clark *et al.*, *N Engl J Med* 342, 469-74, 2000; Roberts *et al.*, *N Engl J Med* 336, 605-10, 1997; The Neonatal Inhaled Nitric Oxide Study Group. *N Engl J Med* 336, 597-604, 1997). A recent randomized placebo-controlled trial in premature infants with respiratory distress syndrome indicated that treatment with inhaled NO reduced the combined endpoint of death and chronic lung disease (Schreiber *et al.*, *N Engl J Med* 349, 2099-107, 2003).

Despite the encouraging results regarding treatment of persistent pulmonary hypertension of the newborn with inhaled NO, the therapy does have several significant limitations (Martin, *N Engl J Med* 349, 2157-9, 2003): considerable cost (Jacobs *et al.*, *Crit Care Med* 30, 2330-4, 2002; Pierce *et al.*,. *Bmj* 325, 336, 2002; Subhedar *et al.*, *Lancet* 359, 1781-2, 2002; Angus *et al.*, *Pediatrics* 112, 1351-60, 2003), technical difficulties involved in adapting NO delivery systems for neonatal transport (Kinsella *et al.*, *Pediatrics* 109, 158-61, 2002), and the lack of availability in small community

hospitals and developing countries. In addition, NO reacts with oxygen, forming the toxic nitrogen dioxide, and thus must be stored and delivered in nitrogen at high flow rates. The gas and delivery systems are costly and the requisite delivery technology is not universally available. Therefore, alternative NO-based therapies for the treatment of pulmonary hypertension are highly desirable.

The relationship between nitrite and nitric oxide has been appreciated for close to a century, with Haldane and later Hoagland recognizing that iron-nitrosylated myoglobin (NO bound to heme) formed as an end-product during nitrite-based meat curing (Gladwin, *J Clin Invest* 113, 19-21, 2004). More than fifty years ago, Furchgott and Bhadrakom reported that nitrite vasodilated aortic ring preparations *in vitro* (Furchgott & Bhadrakom, *J Pharmacol Exp Ther* 108, 129-43, 1953); this observation was later explored by Ignarro's group in experiments evaluating the role of soluble guanylyl cyclase in endothelium-dependent vasodilation (Ignarro *et al.*, *J Pharmacol Exp Ther* 218, 739-49, 1981). However, the high concentrations of nitrite, typically in the millimolar range, required to elicit vasodilation in aortic ring *in vitro* bioassays precluded consideration of nitrite as a physiological vasodilator (Lauer *et al.*, *Proc Natl Acad Sci U S A* 98, 12814-9, 2001; Pawloski, *N Engl J Med* 349, 402-5; author reply 402-5, 2003; McMahon, *N Engl J Med* 349, 402-5; author reply 402-5, 2003).

Two decades later, in human physiological studies, we observed artery-to-vein differences for nitrite across the human forearm with increased extraction occurring during NO inhalation and exercise stress with concomitant NO synthase inhibition (Gladwin *et al.*, *Proc Natl Acad Sci U S A* 97, 11482-7, 2000). This finding suggested that nitrite was being metabolized across the forearm with increased consumption during exercise. Based on these observations along with data from a number of investigators that identified mechanisms for non-enzymatic (nitrite disproportionation) (Zweier *et al.*, *Nat Med* 1, 804-9, 1995) and enzymatic (xanthine oxidoreductase) (Zweier *et al.*, *Nat Med* 1, 804-9, 1995; Millar *et al.*, *FEBS Lett* 427, 225-8, 1998; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004; Li *et al.*, *J Biol Chem*, 279(17):16939-16946, 2004) reduction of nitrite to NO, we hypothesized that nitrite is reduced *in vivo* to NO in tissues under conditions of low P_{O_2} or pH. We found support for this hypothesis in studies of normal human volunteers wherein nitrite infusion into the forearm resulted in marked vasodilation even under basal conditions at near-physiological nitrite concentrations (Example 1; Cosby *et al.*, *Nat Med* 9, 1498-505, 2003). The mechanism of this vasodilation was consistent with a reaction of nitrite with deoxygenated hemoglobin to form NO, methemoglobin (Cosby *et al.*, *Nat Med* 9, 1498-505, 2003; Nagababu *et al.*, *J Biol Chem* 278, 46349-56, 2003) and other NO adducts.

This nitrite reductase activity of deoxyhemoglobin was extensively characterized by Doyle and colleagues in 1981 (Doyle *et al.*, *J Biol Chem* 256, 12393-8, 1981): nitrite appears to react with deoxyhemoglobin and a proton to form NO and methemoglobin. Such chemistry is ideally suited for hypoxic generation of NO from nitrite, as the reaction is enhanced by hemoglobin deoxygenation and acid, providing a graded production of NO from nitrite linked to physiological changes in oxygen and pH/CO_2. The observation in this current example that inhaled nitrite generates iron-nitrosyl-hemoglobin, exhaled NO gas, and produces vasodilation in proportion to decreasing levels of

oxygenation and pH further indicates that nitrite is a bioavailable storage pool of NO and that hemoglobin may have a physiological function as a nitrite reductase, potentially contributing to hypoxic vasodilation (see Example 1). In addition to these mechanistic considerations, this example supports another therapeutic application of nitrite, extending beyond its well-established role in the treatment of cyanide poisoning.

We show herein (Example 3) that this biochemical reaction can be harnessed for the treatment of neonatal pulmonary hypertension, an NO-deficient state characterized by pulmonary vasoconstriction, right-to-left shunt pathophysiology, ventilation/perfusion inhomogeneity and systemic hypoxemia. We delivered inhaled sodium nitrite by aerosol to newborn lambs with hypoxic and normoxic pulmonary hypertension. Inhaled nitrite elicited a rapid and sustained reduction (~60%) in hypoxia induced pulmonary hypertension, a magnitude approaching that of the effects of 20 ppm NO gas inhalation and which was associated with the immediate appearance of increasing levels of NO in expiratory gas. Pulmonary vasodilation elicited by aerosolized nitrite was deoxyhemoglobin- and pH-dependent and was associated with increased blood levels of hemoglobin iron-nitrosylation. Significantly, from a therapeutic standpoint, short term delivery of nitrite, dissolved in saline, via nebulization produced selective and sustained pulmonary vasodilation with no appreciable increase in blood methemoglobin levels. These data support the paradigm that nitrite is a vasodilator acting via conversion to NO, a process coupled to hemoglobin deoxygenation and protonation, and further evince a novel, simple and inexpensive potential therapy for neonatal pulmonary hypertension.

Aerosolized nitrite is an effective vasodilatory in the described newborn lamb model (Example 3). It can be readily administered by nebulization, and appears to exhibit a wide therapeutic-to-safety margin, with limited systemic hemodynamic changes and methemoglobin production. This presents an attractive therapeutic option to inhaled NO. Nitrite is an ideal "NO producing" agent in that it 1) is a naturally occurring compound in blood, alveolar lining fluid, and tissue, and 2) has no parent-compound leaving group, such as the diazenium diolates, that requires extensive toxicological study prior to translation to human disease.

Inhaled nitrite is a potent and selective vasodilator of pulmonary circulation of the newborn lamb. This further supports the paradigm that nitrite is an NO-dependent vasodilator whose bioactivation is coupled to hemoglobin deoxygenation and protonation. This has clinical applications in veterinary and medical situations, including pulmonary hypertension and other pulmonary syndromes with apparent NO deficiencies. Based on the data presented herein, it is believed that inhaled nitrite will have efficacy in all known and tested applications of inhaled NO.

Prevention of Cerebral Artery Vasospasm after Subarachnoid Hemorrhage

Further, it has been discovered that nitrite infusion can be used to prevent cerebral artery vasospasm after aneurismal hemorrhage (Example 4). Subarachnoid hemorrhage (SAH) due to the rupture of intracranial aneurysms affects 28,000 Americans annually. Almost 70% of patients with aneurysmal SAH develop severe spasm of the cerebral arteries on the seventh day after SAH.

Despite aggressive medical therapy, neurological deficits resulting from vasospasm continue to be a major cause of morbidity and mortality. Although the etiology of cerebral vasospasm is poorly understood, there is increasing evidence that erythrocyte hemolysis in the cerebrospinal fluid and decreased availability of nitric oxide (NO), a potent vasodilator, plays a significant role. Reversal of vasospasm by NO or NO prodrugs has been documented in several animal models.

Delayed cerebral vasospasm (DCV) remains the single cause of permanent neurological deficits or death in at least fifteen percent of patients following otherwise successful endovascular or surgical treatment for ruptured intracranial aneurysm. Decreased bioavailability of nitric oxide (NO) has been mechanistically associated with the development of DCV. A primate model system for cerebral artery vasospasm was used to determine whether infusions of nitrite, a naturally occurring anion that reacts with deoxyhemoglobin to form NO and S-nitrosothiol, might prevent DCV via reactions with perivascular hemoglobin.

As described in Example 4, nitrite infusions (45 mg/kg and 60 mg/kg per day) that produced blood levels of nitrite ranging from 10-60 microM with no clinically significant methemoglobin formation (<5%) were associated with increases in plasma cerebrospinal fluid nitrite and modest increases in blood methemoglobin concentrations (2% or less) without systemic hypotension, and significantly reduced the severity of vasospasm (Figures 15 and 16). No animals infused with sodium nitrite developed significant vasospasm; mean reduction in the R MCA area on day 7 after SAH was 8±9% versus 45±5%; $P < 0.001$) Pharmacological effects of nitrite infusion were associated with bioconversion of cerebrospinal fluid nitrite to S-nitrosothiol, a potent vasodilating NO donor intermediate of nitrite bioactivation. There was no clinical or pathological evidence of nitrite toxicity.

Subacute sodium nitrite infusions prevent DCV in a primate model of SAH, and do so without toxicity. These data evince a novel, safe, inexpensive, and rationally designed therapy for DCV, a disease for which no current preventative therapy exists.

The results presented herein suggest that sodium nitrite therapy may prevent tissue injury produced by metabolic products of hemoglobin, either by vascular spasm, or by other mechanisms of tissue injury by these metabolic products.

Treatment or Amelioration of Gestational or Fetal Cardiovascular Malconditions

Based on results presented herein, it is believed that nitrite, particularly pharmaceutically acceptable salts of nitrite as described herein, can be used to treat hypertension and preeclampsia during pregnancy. Such therapy would include action of nitrites on spastic and diseased blood vessels within the placenta.

Also suggested are methods for treating fetuses in utero, particularly those afflicted with cardiovascular anomalies, hypertension, and misdirected blood flow. It is believed that it may be possible to add nitrites to the amniotic fluid, and thus indirectly to the fetus, to achieve vasodilation and redistribution of blood flow before birth. By this means, fetal cardiovascular system development and function could be altered, for instance with promotion of blood flow to the brain

and heart. To be effective longer term, it is envisioned that embodiments of such fetal therapy would include the introduction of one or more mini-osmotic pumps, containing nitrite (*e.g.*, sodium nitrite), into the amniotic cavity to thereby achieve sustained, slow release. For instance, such minipumps could be used to achieve sustained release throughout days and weeks of pregnancy.

Also suggested are methods for treating fetuses in whom plasma nitrite levels may be depressed by immune incompatibility and associated hemolytic anemias. Such fetal treatment may be extended into the neonatal period. Administrated in the fetal period may include implantation of nitrite-charged osmotic minipumps into the amniotic cavity and could include aerosol inhalation after birth.

VIII. Formulations and Administration

Nitrites, including their salts, are administered to a subject in accordance to methods provided herein, in order to decrease blood pressure and/or increase vasodilation in a subject. Administration of the nitrites in accordance with the present disclosure may be in a single dose, in multiple doses, and/or in a continuous or intermittent manner, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the nitrites may be essentially continuous over a preselected period of time or may be in a series of spaced doses. The amount administered will vary depending on various factors including, but not limited to, the condition to be treated and the weight, physical condition, health, and age of the subject. Such factors can be determined by a clinician employing animal models or other test systems that are available in the art.

To prepare the nitrites, nitrites are synthesized or otherwise obtained and purified as necessary or desired. In some embodiments of the disclosure, the nitrite is a pharmaceutically-acceptable salt of nitrite, for example, sodium nitrite. In some embodiments of the disclosure, the nitrite is not ethyl nitrite. In some embodiments of the disclosure, the sodium nitrite is not on a medical devise, for example, not on a stent. In some embodiments of the disclosure, the nitrite is not in the form of a gel. The nitrites can be adjusted to the appropriate concentration, and optionally combined with other agents. The absolute weight of a given nitrite included in a unit dose can vary. In some embodiments of the disclosure, the nitrite is administered as a salt of an anionic nitrite with a cation, for example, sodium, potassium, or arginine.

One or more suitable unit dosage forms including the nitrite can be administered by a variety of routes including topical, oral (for instance, in an enterically coated formulation), parenteral (including subcutaneous, intravenous, intramuscular and intraperitoneal), rectal, intraamnitic, dermal, transdermal, intrathoracic, intrapulmonary and intranasal (respiratory) routes.

The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods known to the pharmaceutical arts. Such methods include the step of mixing the nitrite with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the

product into the desired delivery system. By "pharmaceutically acceptable" it is meant a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious or unsuitably harmful to the recipient thereof. The therapeutic compounds may also be formulated for sustained release, for example, using microencapsulation (see WO 94/ 07529, and U.S. Patent No. 4,962,091).

The nitrites may be formulated for parenteral administration (*e.g.*, by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusion containers or in multi-dose containers. Preservatives can be added to help maintain the shelve life of the dosage form. The nitrites and other ingredients may form suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the nitrites and other ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, *e.g.*, sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable carriers and vehicles that are available in the art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol," polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol," isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

It is possible to add other ingredients such as antioxidants, surfactants, preservatives, film-forming, keratolytic or comedolytic agents, perfumes, flavorings and colorings. Antioxidants such as t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol and its derivatives can be added.

The pharmaceutical formulations of the present disclosure may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are available in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present disclosure include water and physiologically acceptable buffered saline solutions, such as phosphate buffered saline solutions. Merely by way of example, the buffered solution can be at a pH of about 6.0-8.5, for instance about 6.5-8.5, about 7-8.

The nitrites can also be administered via the respiratory tract. Thus, the present disclosure also provides aerosol pharmaceutical formulations and dosage forms for use in the methods of the disclosure. In general, such dosage forms include an amount of nitrite effective to treat or prevent the clinical symptoms of a specific condition. Any attenuation, for example a statistically significant attenuation, of one or more symptoms of a condition that has been treated pursuant to the methods of the present disclosure is considered to be a treatment of such condition and is within the scope of the disclosure.

For administration by inhalation, the composition may take the form of a dry powder, for example, a powder mix of the nitrite and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, *e.g.*, gelatin or blister packs from which the powder may be administered with the aid of an inhalator, insufflator, or a metered-dose inhaler (see, for example, the pressurized metered dose inhaler (MDI) and the dry powder inhaler disclosed in Newman, S. P. in *Aerosols and the Lung*, Clarke, S. W. and Davia, D. eds., pp. 197-224, Butterworths, London, England, 1984).

Nitrites may also be administered in an aqueous solution, for example, when administered in an aerosol or inhaled form. Thus, other aerosol pharmaceutical formulations may include, for example, a physiologically acceptable buffered saline solution. Dry aerosol in the form of finely divided solid compound that is not dissolved or suspended in a liquid is also useful in the practice of the present disclosure.

For administration to the respiratory tract, for example, the upper (nasal) or lower respiratory tract, by inhalation, the nitrites can be conveniently delivered from a nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may include a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Nebulizers include, but are not limited to, those described in U.S. Patent Nos. 4,624,251; 3,703,173; 3,561,444; and 4,635,627. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co. (Valencia, CA). For intra-nasal administration, the therapeutic agent may also be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker). The nitrites may also be delivered via an ultrasonic delivery system. In some embodiments of the disclosure, the nitrites may be delivered via an endotracheal tube. In some embodiments of the disclosure, the nitrites may be delivered via a face mask.

The present disclosure further pertains to a packaged pharmaceutical composition such as a kit or other container. The kit or container holds a therapeutically effective amount of a pharmaceutical composition of nitrite and instructions for using the pharmaceutical composition for treating a condition.

IX. Combination Therapies

Furthermore, the nitrite may also be used in combination with other therapeutic agents, for example, pain relievers, anti-inflammatory agents, antihistamines, and the like, whether for the conditions described or some other condition. By way of example, the additional agent is one or more selected from the list consisting of penicillin, hydroxyurea, butyrate, clotrimazole, arginine, or a phosphodiesterase inhibitor (such as sildenafil).

Generally, it is believed that therapies that have been suggested or demonstrated to be effective when combined with NO therapy, may also be effective when combined with nitrite administration. All combination therapies that have been are being studied with NO therapy (inhaled or otherwise) are likely to be worthy of study in combination with nitrite therapy. See, for instance, Uga et al., *Pediatr. Int.* 46 (1): 10-14, 2004; Gianetti *et al.*, *J Thorac. Cardiov. Sur.* 127 (1): 44-50, 2004; Stubbe et al., *Intens. Care Med.* 29 (10): 1790-1797, 2003; Wagner *et al.*, Eur. Heart J 23: 326-326 Suppl. 2002; Park *et al.*, Yonesi Med J 44 (2):219-226, 2003; Kohele, *Israel Med. Assoc. J.* 5:19-23, 2003, for discussions of combination therapies used with NO.

Furthermore, pharmaceutically-acceptable nitrite salts (such as, for instance, sodium nitrite) may be used in combinations with drugs and agents that limit the elimination rate of administered nitrites. This combination could serve to prolong the duration of action of nitrite and would include antagonists and inhibitors of enzymes affecting the elimination of nitrites or their conversion to NO.

Alternatively, the nitrite may be used in combinations with drugs and agents that augment the action of nitrites. This combination could serve to increase the strength of responses to administered nitrites.

Recombinant tissue plasminogen activator (rt-PA) and urokinase are the only drugs that have proven to open occluded brain arteries in ischemic stroke. It is believed possible that using nitrite via quenching oxygen free radicals produced in response to reperfusion may provide an additional beneficial effect.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the invention to the particular features or embodiments described.

Example 1

Nitrite has vasodilatory properties *in vivo*

This example provides a demonstration that nitrite, administered by infusion to the forearm of human subjects, is an effective vasodilator.

Methods

Human subjects protocol.

The protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and informed consent was obtained from all volunteer subjects. Nine men and nine women, with an average age of 33 years (range 21 - 50 years), participated in the study. An additional 10 subjects returned three-six months later for a second series of experiments with low dose nitrite infusion. Volunteers had a normal hemoglobin concentration, and all were in excellent general health without risk factors for endothelial dysfunction (fasting blood sugar >120 mg/dL, low-density lipoprotein cholesterol >130 mg/dL, blood pressure >145/95 mmHg, smoking within two years, cardiovascular disease, peripheral vascular disease, coagulopathy, or any other disease

predisposing to vasculitis or Raynaud's phenomenon). Subjects with G6PD deficiency, known cytochrome B5 deficiency or a baseline methemoglobin level > 1% were excluded (no screened subjects met these exclusion criteria). Lactating and pregnant females were excluded (one subject with positive HCG levels was excluded). No volunteer subject was allowed to take any medication (oral contraceptive agents allowed), vitamin supplements, herbal preparations, nutriceuticals or other "alternative therapies" for at least one month prior to study and were not be allowed to take aspirin for one week prior to study.

Forearm blood flow measurements

Brachial artery and antecubital vein catheters were placed into the arm, with the intra-arterial catheter connected to a pressure transducer for blood pressure measurements and an infusion pump delivering normal saline at 1 mL/min. After 20 minutes of rest, baseline arterial and venous blood samples were obtained and forearm blood flow measurements were made by strain gauge venous-occlusion plethysmography, as previously reported (Panza *et al.*, *Circulation, 87*, 1468-74, 1993). A series of 7 blood flow measurements were averaged for each blood flow determination. A series of measurements termed Parts I and II were performed in randomized order to minimize a time effect on the forearm blood flow response during nitrite infusion.

Measurement of blood flow and forearm nitrite extraction during NO blockade and repetitive exercise

Part I: Following 20 minutes of 0.9% NaCl (saline) solution infusion at 1 mL/min into the brachial artery, arterial and venous blood samples were obtained for the assays described below and forearm blood flow measured. Exercise was performed by repetitive hand-grip at one-third of the predetermined maximum grip strength using a hand-grip dynamometer (Technical Products Co.) (Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 9943-8, 2000; Gladwin *et al.*, *Proc Natl Acad Sci U S A, 97*, 11482-11487, 2000; Cannon *et al.*, *J Clin Invest, 108*, 279-87, 2001). Each contraction lasted for 10 seconds followed by relaxation for 5 seconds. Following 5 minutes of exercise, forearm blood flow measurements were obtained during relaxation phases of exercise, and arterial and venous samples collected. Following a 20-minute rest period with continued infusion of saline into the brachial artery, repeated baseline blood samples and forearm blood flow measurements were obtained. L-NMMA was then infused at a rate of 1 mL/min (8 μmol/min) into the brachial artery. Following 5 minutes of L-NMMA infusion, forearm blood flow was measured, and arterial and venous blood samples obtained. Forearm exercise was then initiated in that arm during continued L-NMMA infusion. Forearm blood flow was measured and blood samples obtained after 5 minutes of exercise during continued L-NMMA infusion (Figure 1).

Part II: After a 30 minute rest period with continued infusion of saline, baseline measurements were obtained, the saline infusion was then stopped, and infusion of nitrite ($NaNO_2$ 36 μmol/ml in 0.9% saline) at 1 ml/min was started. Sodium nitrite for use in humans was obtained from Hope Pharmaceuticals (300 mg in 10 ml water) and 286 mg was diluted in 100 ml 0.9% saline

by the Pharmaceutical Development Service to a final concentration of 36 μmol/ml. For the final 9 subjects studied, 0.01-0.03 mM sodium bicarbonate was added to the normal saline, so as to titrate pH to 7.0-7.4. The nitrite solution was light protected and nitrite levels and free NO gas in solution measured by reductive chemiluminescence after all experiments (Gladwin *et al.*, *J Biol Chem, 21*, 21, 2002). Only 50.5 ± 40.5 nM NO was present in nitrite solutions and was unaffected by bicarbonate buffering. There was no correlation between NO levels in nitrite solutions and blood flow effects of nitrite (r = -0.23; P=0.55). After 5 minutes of nitrite infusion, forearm blood flow measurements and blood samples were obtained, with brief interruption of the nitrite infusion to obtain the arterial sample. With continued nitrite infusion, exercise was performed as described previously, with forearm blood flow measurements and blood samples obtained as described above. The nitrite infusion was stopped and saline infusion re-started during the subsequent 30-minute rest period. Following second baseline measurements, the nitrite infusion was re-initiated, along with L-NMMA at 8 μmol/min. Five minutes later, forearm blood flow measurements were performed and blood samples obtained followed by 5 minutes of exercise with continuation of nitrite and L-NMMA infusions. Final forearm blood flow measurements and blood samples obtained. At all time points during part II, blood samples were obtained from the contralateral arm antecubital vein for determination of methemoglobin and systemic levels of NO-modified hemoglobin (Figure 2, 3, and 4). The total dose of sodium nitrite infused was 36 μmol/min x 15 minutes x 2 infusions = 1.08 mmol = 75 mg (MW $NaNO_2$ = 69).

In additional studies in 10 subjects the same stages of Parts I and II protocol were followed with infusion of low dose nitrite ($NaNO_2$ 0.36 μmol/ml in 0.9% saline, infused at 1 ml/min).

Arterial and venous pH, pO_2, and pCO_2, were measured at the bedside using the i-STAT system (i-STAT Corporation, East Windsor, NJ) and methemoglobin concentration and hemoglobin oxygen saturation measured by co-oximetry.

Measurement of red blood cell S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin.

S-nitroso-hemoglobin is unstable in the reductive red blood cell environment and rapidly decays in a temperature and redox dependent fashion, independent of oxygen tension (Gladwin *et al.*, *J Biol Chem,* 21:21, 2002). To stabilize the S-nitroso-hemoglobin for measurement, the red blood cell must be rapidly oxidized with ferricyanide. Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" of PBS containing 1% NP-40 (to solubilize membranes), 8 mM NEM (to bind free thiol and prevent artefactual S-nitrosation), 0.1 mM DTPA (to chelate trace copper), and 4 mM ferricyanide and cyanide (to stabilize S-nitrosohemoglobin and prevent artefactual ex-vivo iron-nitrosylation during processing). The samples were desalted across a 9.5 mL bed volume Sephadex G25 column to eliminate nitrite and excess reagents and partially purify hemoglobin (99% hemoglobin preparation). The hemoglobin fraction was quantified by the method of Drabkin, and hemoglobin fractions reacted with and without mercuric chloride (1:5 $HgCl_2$:heme ratio- used to differentiate S-nitrosothiol which

is mercury labile versus iron-nitrosyl which is mercury stable) and then in 0.1 M HCL/0.5%sulfanilamide (to eliminate residual nitrite; Marley *et al.*, *Free Radic Res, 32*, 1-9, 2000). The samples were then injected into a solution of tri-iodide (I_3^-) in-line with a chemiluminescent nitric oxide analyzer (Sievers, Model 280 NO analyzer, Boulder, CO). The mercury stable peak represents iron-nitrosyl-hemoglobin. This assay is sensitive and specific for both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin to 5 nM in whole blood (0.00005% S-NO per heme) (Gladwin *et al.*, *J Biol Chem, 21*, 21, 2002).

Analysis was initially performed using red blood cell pellet, however, despite placing the sample in ice and immediately separating plasma from erythrocyte pellet, NO formed in the venous blood *ex vivo*. To measure the true *in vivo* levels, whole blood was mixed at the bedside 1:10 in the "NO-hemoglobin stabilization solution". Plasma S-nitroso-albumin formation was negligible during nitrite infusion so this bedside whole blood assay was used to limit processing time and thus more accurately characterize the *in vivo* chemistry. In a series of validation experiments, both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were stable in the "NO-hemoglobin stabilization solution" for 20 minutes at room temperature with no artifactual formation or decay of NO-modified species (n=6).

Chemiluminescent detection of NO gas released from deoxyhemoglobin and deoxygenated erythrocytes following nitrite addition.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was mixed with 5 mL of 660 and 1000 μM deoxygenated erythrocytes in a light protected reaction vessel purged with helium or oxygen (both 21% and 100%) in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO). After allowing equilibration for 5 minutes, nitrite was injected and the rate of NO production measured. Nitrite was injected into PBS as a control and into 100 μM hemoglobin to control for the hemolysis in the 660 and 1000 μM deoxygenated erythrocyte solutions. At the end of all experiments the visible absorption spectra of the supernatant and erythrocyte reaction mixture was analyzed and hemoglobin composition deconvoluted using a least-squares algorithm. There was less than 100 μM hemolysis in the system, no hemoglobin denaturation, and significant formation of iron-nitrosyl-hemoglobin. The NO production from erythrocyte suspensions exceeded that produced from the hemolysate control, consistent with NO export from the erythrocyte.

Statistical analysis.

An *a priori* sample size calculation determined that 18 subjects would be necessary for the study to detect a 25% improvement in forearm blood flow during nitrite infusion when forearm NO synthesis had been inhibited by L-NMMA compared with normal saline infusion control values (alpha=0.05, power=0.80). Two-sided P values were calculated by paired t-test for the pair-wise comparisons between baseline and L-NMMA infusion values, between baseline and exercise values, and between nitrite and saline control values at comparable time-points of the study. Repeated

measures ANOVA were performed for artery-to-vein gradients of NO species during basal, L-NMMA infusion, and exercise conditions. Measurements shown are mean ± SEM.

Results and Discussion

Eighteen healthy subjects (9 males, 9 females; age range 21 to 50 years) were enrolled in a physiological study to determine if nitrite is a vasodilator and to examine nitrite's *in vivo* chemistry. Part I of the protocol was designed to measure the normal hemodynamic and metabolic responses to exercise and to inhibition of NO synthesis within the forearm as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion. Initial baseline measurements included a mean blood pressure of 85.6 ± 3.7 mm Hg and forearm blood flow of 4.0 ± 0.3 ml/min per 100 mL tissue (Figure 1A). Repetitive hand-grip forearm exercise increased blood flow approximately 600% over resting values, and significantly decreased ipsilateral venous hemoglobin oxygen saturation, pO_2, and pH, consistent with increased oxygen consumption and CO_2 generation. Following a 20-minute rest period, repeat hemodynamic measurements showed an approximate 10% higher forearm blood flow, but no change in systemic blood pressure or forearm venous hemoglobin oxygen saturation, pO_2 and pH values compared with the initial baseline values (Figure 1B). The NO synthase inhibitor L-NMMA was then infused into the brachial artery at 8 μmol/min for 5 minutes, significantly reducing forearm blood flow by approximately 30% and significantly reducing venous hemoglobin oxygen saturation, pO_2 and pH values. Repeated forearm exercise during continued L-NMMA infusion increased blood flow, but to a significantly lower peak value compared with exercise alone ($P<0.001$). In addition, hemoglobin oxygen saturation, pO_2 and pH were significantly lower during exercise with L-NMMA than with exercise without regional NO synthase inhibition ($P<0.001$, $P<0.005$ and $P=0.027$, respectively). Mean arterial blood pressure was unchanged during all components of Part I of the protocol.

Figure 1 depicts hemodynamic and metabolic measurements at baseline and during exercise, without (Figure 1A) and with (Figure 1B) inhibition of NO synthesis in 18 subjects. Mean arterial pressure (MAP), forearm blood flow (FBF), and venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2), and pH are shown for all experimental conditions. These interventions and measurements (part I of the protocol) served as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion.

To determine whether nitrite has vasoactivity in humans, in Part II of the protocol sodium nitrite in bicarbonate-buffered normal saline (final concentration 36 μmol/ml) was infused into the brachial arteries of these 18 subjects to achieve an estimated intravascular concentration of approximately 200 μM (Lauer *et al.*, *Proc Natl Acad Sci U S A, 98*, 12814-9, 2001). Following repeat baseline measurements and infusion of sodium nitrite at 1 mL/min for 5 minutes, nitrite levels in the ipsilateral antecubital vein increased from 3.32 ± 0.32 to 221.82 ± 57.59 μM (Figure 2A). Forearm blood flow increased 175% over resting values; venous hemoglobin oxygen saturation, pO_2 and pH levels significantly increased over pre-infusion values, consistent with increased perfusion of the forearm.

Systemic levels of nitrite were 16 μM as measured in the contralateral arm and were associated with a systemic effect of decreased mean blood pressure of approximately 7 mm Hg. Consistent with immediate NO generation from nitrite during an arterial-to-venous transit, iron-nitrosylated-hemoglobin in the ipsilateral antecubital vein increased from 55.7 ± 11.4 to 693.4 ± 216.9 nM during the nitrite infusion. During forearm exercise with continuation of the nitrite infusion, blood flow increased further, with evidence of metabolic stress by virtue of reduction in forearm venous hemoglobin oxygen saturation, pO_2 and pH levels from baseline values. Venous nitrite levels declined, consistent with increased blood flow to the forearm diluting the concentration of infused nitrite. Despite decreasing forearm nitrite concentrations during exercise, iron-nitrosyl-hemoglobin levels increased (Figure 2A).

Following cessation of nitrite infusion and substitution of saline as the intra-arterial infusate for 30 minutes, repeat baseline measurements showed persistent elevations in systemic levels of nitrite, iron-nitrosyl-hemoglobin and methemoglobin (Figure 2B) over values obtained prior to the infusion of nitrite almost one hour before. In addition, persistence of a vasodilator effect was also apparent, as forearm blood flow was significantly higher (4.79 ± 0.37 versus 3.94 ± 0.38 mL/min per 100 mL tissue, P=0.003) and systemic blood pressure significantly lower (82.1 ± 3.7 versus 89.2 ± 3.5 mm Hg, P=0.002) than initial pre-nitrite infusion values. During re-infusion into the brachial artery of sodium nitrite 36 μmol/ml, combined with L-NMMA 8 μmol/min in order to again inhibit regional synthesis of NO, similar vasodilator effects of nitrite on resting and exercise forearm blood flow were seen as during nitrite infusion without L-NMMA (Figure 2B). This stands in contrast to the vasoconstrictor effect of NO synthase inhibition with L-NMMA observed in Part I of the protocol (Figure 1B). Venous nitrite and iron-nitrosyl-hemoglobin levels followed similar patterns during NO inhibition as during the initial nitrite infusion.

Figure 2 depicts the effects of infusion of sodium nitrite ($NaNO_2$) in bicarbonate-buffered normal saline (0.9%; final concentration 36 μmol/ml) into the brachial arteries of 18 healthy subjects at 1 ml/min for 5 minutes at baseline and continued during exercise. Figure 2A depicts the effects without inhibition of NO synthesis. Figure 2B depicts the effects with inhibition of NO synthesis. Values for mean arterial blood pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2) and pH, venous nitrite, venous iron-nitrosyl-hemoglobin and venous methemoglobin are shown for all experimental interventions.

As a test of the physiological relevance of vascular nitrite as a vasodilator, nitrite concentrations were decreased by 2-logs to 400 nmol/mL. An infusion of 1 mL/min for five minutes in 10 subjects significantly increased forearm blood flow in all ten subjects from 3.49 ± 0.24 to 4.51 ± 0.33 ml/min per 100 mL tissue (Figure 3A; P=0.0006). Blood flow significantly increased at rest and during NO synthase inhibition with and without exercise (Figure 3B; P<0.05 during all conditions). Mean venous nitrite levels increased from 176 ± 17 nM to 2564 ± 462 nM following a five-minute infusion and exercise venous nitrite levels decreased to 909 ± 113 nM (secondary to dilutional effects of increased flow during exercise; Figure 3C). Again, the vasodilator effects of nitrite were paralleled with an observed formation of both iron-nitrosyl-hemoglobin and S-nitroso-

hemoglobin across the forearm circulation (Figure 3D; described below). These data indicate that basal levels of nitrite, from 150-1000 nM in plasma to 10,000 nM in vascular tissue, contribute to resting vascular tone and hypoxic vasodilation.

Figure 3 depicts the effects of infusion of low-dose sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. Figure 3A depicts forearm blood flow at baseline and following a five-minute in fusion of $NaNO_2$ (0.36 μmol/ml in 0.9% saline, infused at 1 ml/min). Figure 3B depicts forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. Figure 3C depicts venous levels of nitrite from the forearm circulation at the time of blood flow measurements. Figure 3D depicts venous levels of S-nitroso-hemoglobin (S-NO) and iron-nitrosyl-hemoglobin (Hb-NO) at baseline and following nitrite infusion during exercise stress.

The vasodilatory property of nitrite during basal blood flow conditions, when tissue pO_2 and pH are not exceedingly low, was unexpected. These results indicate that the previously hypothesized mechanisms for nitrite reduction, nitrite disproportionation and xanthine oxidoreductase activity, both of which require extremely low pO_2 and pH values not typically encountered in normal physiology, are complemented *in vivo* by additional factors that serve to catalyze nitrite reduction. While ascorbic acid and other reductants, present in abundance in blood, can provide necessary electrons for nitrous acid reduction, such that the reaction might occur at physiologically attainable pH levels, it is herein reported that deoxyhemoglobin effectively reduces nitrite to NO, within one half-circulatory time. This mechanism provides a graded production of NO along the physiological oxygen gradient, tightly regulated by hemoglobin oxygen desaturation.

Intravascular formation of NO and S-nitrosothiol by reaction of nitrite with intraerythrocytic deoxyhemoglobin

Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" and the iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin content determined by tri-iodide-based reductive chemiluminescence and electron paramagnetic resonance spectroscopy as described in Methods. The baseline levels of S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were at the limits of detection (<50 nM or 0.0005% NO per heme) with no artery-to-vein gradients. Following nitrite infusion in Part II of the protocol venous levels of both iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin rose strikingly (Figure 4A). The formation of both NO-hemoglobin adducts occurred across the vascular bed, a half-circulatory time of less than 10 seconds. The rate of NO formation, measured as iron-nitrosyl and S-nitroso-hemoglobin and quantified by subtraction of the arterial from the venous levels with the difference being multiplied by blood flow, increased greatly during exercise, despite a significant decrease in the venous concentration of nitrite secondary to increasing blood flow diluting the

regional nitrite concentration (Figure 4A; P=0.006 for iron-nitrosyl-hemoglobin and P=0.02 for S-nitroso-hemoglobin by repeated measures ANOVA).

Figure 4A depicts formation of iron-nitrosyl-hemoglobin (black squares) and S-nitroso-hemoglobin (red circles) during nitrite infusion at baseline, during nitrite infusion and during nitrite infusion with exercise, quantified by subtraction of the arterial from the venous levels and multiplying the result by blood flow. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation in the human circulation during nitrite infusion (for iron-nitrosyl-hemoglobin r=-0.7, p<0.0001, for S-nitroso-hemoglobin r=-0.45, p=0.04) (Figure 4B). Hemoglobin oxygen saturation was measured from the antecubital vein by co-oximetry. Asterix in all figures signify P<0.05 by paired t test or repeated measures analysis of variance.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was reacted with deoxygenated erythrocytes (5 mL volume containing a total of 660 and 1000 μM in heme) in a light protected reaction vessel purged with helium in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO.). As shown in Figure 5A and 5B, the injection of nitrite into a solution of deoxygenated erythrocytes resulted in the liberation of NO into the gas phase. There was no release from nitrite in buffer control under the same conditions, and significantly less NO was released upon nitrite addition to oxygenated erythrocytes (21% and 100% oxygen). The observed rate (determined by the assessment of the area under the curve of increased steady-state NO generation following nitrite injection calculated over 120 seconds) of NO production in the 5 mL reaction volume was consistent with 47 pM NO production per second (corresponding to an estimated 300 to 500 pM NO production per second in whole blood). While NO formation rates in this experimental system may not be extrapolated to rates of NO formation *in vivo*, the experiments are consistent with two important concepts: 1) A fraction of free NO can escape auto-capture by the remaining heme groups; this is likely only possible because nitrite is only converted to NO by reaction with deoxyhemoglobin and its "leaving group" is the met(ferric)heme protein which will limit scavenging and inactivation of NO (Doyle *et al., J Biol Chem, 256*, 12393-12398, 1981); and 2) The rate of NO production is increased under anaerobic conditions, consistent with a nitrite-deoxyhemoglobin reaction.

Example 2

Cytoprotective Effects of Nitrite during Ischemia-reperfusion of the Heart and Liver

As demonstrated in Example 1, nitrite is reduced to NO by reaction with deoxyhemoglobin along the physiological oxygen gradient, a chemistry whose rate is oxygen and pH dependent and that potentially contributes to hypoxic vasodilation. Based on that unexpected discovery, we proposed that hypoxia-dependent NO production from nitrite in ischemic tissue might limit ischemia-reperfusion injury. This example provides a demonstration that infusions of sodium nitrite are effective to provide cytoprotection during ischemia-reperfusion of the heart and liver.

Although reperfusion of ischemic tissues provides oxygen and metabolic substrates necessary for the recovery and survival of reversibly injured cells, reperfusion itself actually results in the acceleration of cellular necrosis (Braunwald *et al.*, *J. Clin. Invest.* 76:1713-1719, 1985). Ischemia-reperfusion is characterized by the formation of oxygen radicals upon reintroduction of molecular oxygen to ischemic tissues resulting in widespread lipid and protein oxidative modifications of cellular proteins, mitochondrial injury, and tissue apoptosis and necrosis (McCord *et al.*, *Adv Myocardiol* 5:183-189, 1985). In addition, following reperfusion of ischemic tissues blood flow may not return uniformly to all portions of the ischemic tissues, a phenomenon that has been termed the "no-reflow" phenomenon (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). Reductions in blood flow following reperfusion are thought to contribute to cellular injury and necrosis (Kloner *et al.*, *J Clin Invest* 54:1496-1508, 1974). The sudden re-introduction of blood into ischemic tissue also results in a dramatic increase in calcium delivery to the previously ischemic tissue (*i.e.*, "calcium paradox") resulting in massive tissue disruption, enzyme release, reductions in high energy phosphate stores, mitochondrial injury, and necrosis (Nayler, *Amer. J. Path.* 102:262, 1981; Shen *et al.*, *Amer. J. Path* 67:417-440, 1972). Recent studies have also indicated that the ischemia-reperfusion injury is also characterized by an inappropriate inflammatory response in the microcirculation resulting in leukocyte-endothelial cell interactions that are mediated by the upregulation of both leukocyte and endothelial cell adhesion molecules (Lefer *et al.*, *Cardiovasc Res* 32:743-751, 1996; Entman *et al.*, *Faseb J* 5:2529-2537, 1991). Intensive research efforts have been focused on ameliorating various pathophysiological components of ischemia-reperfusion injury to limit the extent of tissue injury and necrosis.

NO, NO donors, and NO synthase activation or transgenic over-expression have been shown to exert protective effects on this process in a number of models (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993; Nakanishi *et al.*, *Am J Physiol* 263:H1650-1658, 1992; Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003; Kanno *et al.*, *Circulation* 101:2742-2748, 2000), but in other models appears harmful (Flogel *et al.*, *J Mol Cell Cardiol* 31:827-836. 1999; Menezes *et al.*, *Am J Physiol* 277:G144-151, 1999; Woolfson *et al.*, *Circulation* 91:1545-1551, 1995; Schulz, R. *et al.*, *Cardiovasc Res* 30:432-439, 1995). Evaluation of these studies suggests a critical effect of dose and duration of NO exposure, resulting in a narrow therapeutic safety window for NO in ischemia-reperfusion pathophysiology (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001; Wink *et al.*, *Am J Physiol Heart Circ Physiol* 285:H2264-2276, 2003). An additional limitation is that NO formation from NO synthase requires oxygen as substrate, a molecule whose availability becomes limited during ischemia.

We therefore considered the use of nitrite in this context for the following reasons:

(1) It is a naturally occurring substance with no potentially toxic "leaving group",

(2) it is selectively reduced to NO in tissues with low oxygen tension and low pH (Bryan *et al.*, *Proc Natl Acad Sci USA.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003; Tiravanti *et al.*, *J Biol Chem*

279:11065-11073, 2004; Doyle *et al.*, *J Biol Chem* 256:12393-12398, 1981; Luchsinger *et al.*, *Proc Natl Acad Sci USA* 100:461-466, 2003),

(3) its activation does not require molecular oxygen (Cosby *et al.*, *Nat Med* 9:1498-1505, 2003), and

(4) NO is known to maintain heme proteins in a reduced and liganded state (Herold *et al.*, *Free Radic Biol Med* 34:531-545, 2003; Herold *et al.*, *J Biol Inorg Chem* 6:543-555, 2001; Fernandez *et al.*, *Inorg Chem* 42:2-4, 2003), limit free iron and heme mediated oxidative chemistry (Kanner *et al.*, *Arch Biochem Biophys* 237:314-321, 1985; Kanner *et al.*, *Lipids* 20:625-628, 1985; Kanner *et al.*, *Lipids* 27:46-49, 1992), transiently inhibit cytochome c oxidase and mitochondrial respiration (Torres *et al.*, *FEBS Lett* 475:263-266, 2000; Brown *et al.*, *FEBS Lett* 356:295-298, 1994; Cleeter *et al.*, *FEBS Lett* 345:50-54, 1994; Rakhit *et al.*, *Circulation* 103:2617-2623, 2001), and modulate apoptotic effectors (Mannick *et al.*, *Science* 284:651-654, 1999), all mechanisms that might participate in cytotoxicity following severe ischemia.

We evaluated the effects of nitrite therapy, compared with vehicle and nitrate controls, in well characterized murine models of hepatic and myocardial ischemia-reperfusion injury. The following description provides strong evidence for a profound protective effect of nitrite on cellular necrosis and apoptosis, which is believed to be mediated by a hypoxia-dependent bioconversion of nitrite to NO and nitros(yl)ated proteins.

Materials and Methods

Chemicals and Reagents: Sodium nitrite (S-2252) and sodium nitrate (S-8170) were obtained from the Sigma Chemical Co. (St. Louis, MO). Sodium nitrite and sodium nitrate were dissolved in phosphate buffered saline and the pH was adjusted to 7.4. In all experiments a final volume of 50 μL of sodium nitrite or sodium nitrate were administered to the mice to achieve final concentrations of circulating nitrite of 0.6 to 240 μM assuming a total circulating blood volume of 2mL. Carboxy-PTIO [2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt], a direct intravascular NO scavenger, was utilized to inhibit NO dependent effects following hepatic I/R injury. Carboxy-PTIO (Alexis Biochemicals) was dissolved in phosphate buffered saline and administered intravenously at a dose of 1 mg/Kg in a volume of 50 μL at 30 minutes prior to hepatic ischemia. Zinc(II) Deuteroporphyrin IX-2,4-bisethyleneglycol (ZnDBG) (Alexis Biochemicals) , a heme oxygenase-1 inhibitor was injected i.p. at a dose of 10 mg/Kg in a volume of 50 μL at 30 minutes prior to the induction of hepatic ischemia.

Animals: All of the mice utilized in the present studies were C57BL6/J at 8-10 weeks of age obtained from the Jackson Laboratories (Bar Harbor, ME). In additional experiments of hepatic I/R injury we utilized mice completely deficient (-/-) in endothelial nitric oxide synthase (eNOS). eNOS-/- mice were originally generously donated from Dr. Paul Huang (Mass. General Hospital) and

generated in our breeding colony at LSU-Health Sciences Center. eNOS-/- mice were utilized at 8-10 weeks of age.

Hepatic Ischemia-Reperfusion (I/R) Protocol: The hepatic I/R protocol is depicted in Figure 6A and has been described previously (Hines *et al.*, *Biochem Biophys Res Commun* 284:972-976, 2001; Hines *et al.*, *Am J Physiol Gastrointest Liver Physiol* 284:G536-545, 2001). Mice were anesthetized with the combination of ketamine (100 mg/kg) and zylazine (8 mg/kg) and a midline laparotomy was performed to expose the liver. Mice were then injected with heparin (100 μg/kg, i.p.) to prevent blood clotting. The left lateral and median lobes of the liver were rendered ischemic by completely clamping the hepatic artery and the portal vein using microaneurysm clamps. This experimental model results in a segmental (70%) hepatic ischemia. This method of partial ischemia prevents mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver. The liver was then repositioned in the peritoneal cavity in its original location for 45 minutes. The liver was kept moist using gauze soaked in 0.9% normal saline. In addition, body temperature was maintained at 37°C using a heat lamp and monitoring body temperature with a rectal temperature probe. Sham surgeries were identical except that hepatic blood flow was not reduced with a microaneurysm clamp. The duration of hepatic ischemia was 45 minutes in all experiments, following which the microaneurysm clamps were removed. The duration of hepatic reperfusion was 5 hours in the studies of serum liver transaminase levels (*i.e.*, AST or ALT) and 24 hours for the studies of liver histopathology (such as hepatocellular infarction).

Liver Enzyme Determinations: Serum samples were analyzed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using a spectrophotometric method (Sigma Chemical Co., St. Louis, MO) (Harada *et al.*, *Proc Natl Acad Sci U S A* 100:739-744, 2003). These enzymes are liver specific and are released from the liver during injury (Hines *et al.*, *Biochem Biophys Res Commun* 284:972-976, 2001; Hines *et al.*, *Am J Physiol Gastrointest Liver Physiol* 284:G536-545, 2001).

Liver Histopathology Studies: Histopathology of liver tissue was performed as previously reported (Hines *et al.*, *Biochem Biophys Res Commun* 284:972-976, 2001). Liver tissue was fixed in 10% buffered formalin for 24 hours, embedded in paraffin, and 10 μM sections stained with hematoxylin and eosin. Histopathology scoring was performed in a double blinded manner on random high power fields using the following criteria:

0- no hepatocellular damage,

1- mild injury characterized by cytoplasmic vacuolization and focal nuclear pyknosis,

2- moderate injury with dilated sinusoids, cytosolic vacuolization, and blurring of intercellular borders,

3- moderate to severe injury with coagulative necrosis, abundant sinusoidal dilation, RBC extravasation into hepatic chords, and hypereosinophilia and margination of neutrophils,

4- severe necrosis with loss of hepatic architecture, disintegration of hepatic chords, hemorrhage, and neutrophil infiltration.

Hepatocellular apoptosis was determined using the TUNEL staining kit from Roche according to the manufacturer's recommendations. Briefly, liver tissue from various treatments was fixed in buffered formalin and 10 μm sections were prepared. Sections were permeabilized on ice for 2 minutes and incubated in 50 μL TUNEL solution for 30 minutes at 37°C. Sections were then treated with 50 μL substrate solution for 10 min. and mounted under glass coverslips. The number of apoptotic nuclei was determined from 5 random 40x fields per specimen. A total of six specimens per treatment group (16 slides per group) were analyzed and compared using one-way analysis of variance with Bonferroni's post-testing.

Myocardial Ischemia-Reperfusion (I/R) Protocol: Surgical ligation of the left main coronary artery (LCA) was performed similar to methods described previously (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004). Briefly, mice were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg). The animals were then attached to a surgical board with their ventral side up. The mice were orally intubated with PE-90 polyethylene tubing connected to PE-240 tubing and then connected to a Model 683 rodent ventilator (Harvard Apparatus, Natick, MA). The tidal volume was set at 2.2 milliliters and the respiratory rate was set at 122 breaths per minute. The mice were supplemented with 100% oxygen via the ventilator side port. A median sternotomy was performed using an electric cautery and the proximal left main coronary artery was visualized and completely ligated with 7-0 silk suture mounted on a tapered needle (BV-1 ethicon). In the initial experiments of myocardial infarct size coronary occlusion was maintained for 30-minutes followed by removal of suture and reperfusion for 24 hours. In additional experiments of cardiac function, the proximal LCA was completely occluded for 45 minutes followed by suture removal and reperfusion for 48 hours. In these experiments, two-dimensional echocardiography was performed at baseline and again at 48 hours of reperfusion.

Myocardial Infarct Size Determination: At 24 hours of reperfusion, the mice were anesthetized as described previously, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans Blue dye injection. A median sternotomy was performed and the left main coronary artery was re-ligated in the same location as before Evans Blue dye (1.2 mL of a 2.0% solution, Sigma Chemical Co.) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and serially sectioned along the long axis in five, 1 mm thick sections that were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co.) for 5 minutes at 37°C to demarcate the viable and nonviable myocardium within the risk zone. Each of the five, 1 mm thick myocardial slices were weighed and the areas of infarction, risk, and nonischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image 1.57). All of the procedures for the left ventricular area-at-risk and infarct size determination have been previously described (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004).

Echocardiographic Assessment of Left Ventricular Function: Transthoracic echocardiography of the left ventricle using a 15 MHz linear array transducer (15L8) interfaced with a Sequoia C256 (Acuson) was performed in additional groups of mice (n=9 vehicle and n=10 nitrite)

subjected to 45 minutes of myocardial ischemia and 48 hours of reperfusion. Two-dimensional echocardiography was performed at baseline and at 48 hours of reperfusion as described previously (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003). Ventricular parameters were measured using leading-edge technique. M-mode (sweep speed = 200 mm/sec) echocardiograms were captured from parasternal, short and long-axis 2D views of the left ventricle (LV) at the mid-papillary level. LV percent fractional shortening (FS) was calculated according to the following equation: LV%FS = ((LVEDD-LVESD)/LVEDD) x 100. All data were calculated from 10 cardiaccycles per experiment.

HO-1 Western Blot Analysis of homogenized liver tissue samples (50 μg total protein) was performed using mouse anti-HO-1 mAb (Stressgen, Victoria, BC) at a 1:3,000 dilution and goat anti-mouse secondary Ab (Amersham Biosciences, Piscataway, NJ) at a 1:3,000 dilution.

Blood and Tissue Nitrite Determination: For blood nitrite measurements, 160 μL of whole blood was mixed with 40 μL of a nitrite stabilizing solution containing 80 mM ferricyanide, 20 mM N-ethylmaleimide (NEM), 200 μL diethylenetriaminepentaacetic acid (DTPA), and 0.2% NP-40 (concentrations provided are after mixing with whole blood). The nitrite in whole blood was then measured using tri-idodide-based reductive chemiluminescence as previously described and validated (Gladwin *et al.*, *J Biol Chem* 21:21, 2002; Yang *et al.*, *Free Radic Res* 37:1-10, 2003).

Liver tissue was homogenized using an amended protocol published by Bryan and colleagues (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004). Harvested liver tissue was blotted dry on filter paper, weighed, and homogenized immediately in ice-cold NEM (10 mmol/L)/ DTPA (2 mmol/L) containing buffer (3:1 dilution - w/v). The buffer/tissue mix was then homogenized with a Wheaton glass-glass homogenizer. Tissue homogenates were kept on ice and analyzed within 5 minutes. The homogenate was subsequently either injected directly into triiodine to measure the sum of nitrite, mercury stable (Rx-NO) and mercury-labile (RS-NO) NO-adducts. To determine the levels of specific NO-adducts (Rx-NO and RS-NO), the sample was reacted with and without 5 mM mercuric chloride (RS-NO becomes nitrite in presence of mercuric chloride and Rx-NO is stable) and both treated with acid sulfanilamide (0.5%) to eliminate nitrite.

Statistical Analyses: Data were analyzed by two-way analysis of variance (ANOVA) with post hoc Bonferroni analysis using StatView software version 5.0 (SAS Institute, Carey, North Carolina). Data are reported as means ± standard error of the mean (SEM) with differences accepted as significant when $p < 0.05$.

Results

Intraperitoneal nitrite limits hepatic ischemia-reperfusion (I/R) injury: Intraperitoneal delivery of 1.2 - 480 nmoles of sodium nitrite (0.6 μM to 240 μM estimated final concentration in a 2 mL total blood volume of the mouse) during hepatic ischemia dose-dependently limited serum elevations of liver transaminases, aspartate amino transferase (AST) and alanine amino transferase (ALT) (Figures 6B and 6C), with a peak effect occurring at a calculated systemic concentration of 24 μM (48 nmoles added nitrite). In sharp contrast, treatment with saline or sodium nitrate (48 nmoles)

did not exert any protective effects in the setting of hepatic I/R injury. Additional studies were performed to evaluate the effects of nitrite treatment on hepatocellular injury in mice following *in vivo* hepatic ischemia (45 minutes) and more prolonged reperfusion (24 hours; Figure 6D, 6E, and 6F). The administration of nitrite at a final blood concentration of 24 μM (48 nmoles) significantly reduced hepatocellular injury at 24 hours of reperfusion compared with saline and nitrate treated animals. In addition, nitrite therapy also significantly ($p < 0.001$) attenuated the extent of hepatocellular apoptosis following 45 minutes of hepatic ischemia and 24 hours of reperfusion (Figure 6F). The extent of hepatic cell apoptosis in nitrite treated animals subjected to I/R was similar to that observed in sham operated control animals ($p = NS$).

Intraventricular Nitrite Limits Myocardial Ischemia-Reperfusion Injury: To determine whether the potent cytoprotective effects of nitrite on liver ischemia-reperfusion injury were generalizable to other organ systems, studies were next performed to evaluate the potential cardioprotective effects of acute nitrite therapy in the setting of coronary artery occlusion and reperfusion. The experimental protocol for the myocardial I/R studies is depicted in Figure 7A. Administration of nitrite (48 nmoles) into the left ventricular cavity at 5 minutes prior to reperfusion significantly ($p < 0.001$) limited myocardial infarct size (Figures 7B and 7C) compared to 48 nmoles nitrate treatment. Despite similar myocardial areas-at-risk ($p = NS$ between groups), myocardial infarct size per area-at-risk and per left ventricle were both reduced by 67% with nitrite therapy compared to nitrate.

In additional studies, mice were subjected to 45 minutes of myocardial ischemia and 48 hours of reperfusion to evaluate the effects of nitrite treatment on left ventricular performance (Figures 7D and 7E). In these studies, both myocardial ejection fraction (Figure 7D) and myocardial fractional shortening (Figure 7E) were measured using two-dimensional echocardiography at baseline and following myocardial infarction and reperfusion. Myocardial ejection fraction was similar between the vehicle and nitrite treated study groups at baseline. Following myocardial infarction and reperfusion, ejection fraction was significantly ($p < 0.001$ vs. baseline value) lower in the saline vehicle group, yet remained essentially unchanged in the nitrite treated animals ($p = NS$ vs. baseline). Additionally, ejection fraction was significantly ($p < 0.02$) greater in the nitrite group compared to the vehicle group. Similar observations were made for fractional shortening with no significant group differences at baseline. However, following myocardial infarction and reperfusion, left ventricular fractional shortening was significantly ($p < 0.001$ vs. baseline) depressed in the vehicle group, but not in the nitrite group ($p = NS$ vs. baseline) and was significantly ($p < 0.02$) greater in the nitrite group compared to the vehicle group.

Nitrite-Mediated Cytoprotection is Associated with an Acute Ischemic Reduction of Nitrite to NO and S- and N-nitrosated Proteins within the Liver: Consistent with previously described reduction of nitrite to NO and S-nitrosothiols in a reaction with deoxyhemoglobin and deoxygenated heme proteins (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003; Doyle *et al.*, *J Biol Chem* 256:12393-12398, 1981), one minute after reperfusion the levels of nitrite in the livers of saline (control) treated

mice subjected to ischemia decreased from 1.75 μM to undetectable ($p < 0.001$ vs. sham group) and levels of mercury stable NO modified proteins (likely N-nitrosamines and iron-nitrosyl proteins; RxNO) increased to approximately 750 nM (Figure 8A; $p < 0.001$). Interestingly, with nitrite treatment there was a significant ($p < 0.01$ vs. saline treated controls) increase in post-reperfusion liver levels of nitrite (Figure 8B), S-nitrosothiols (Figure 8C) and N-nitrosamines (Figure 8D) in the nitrite treated mice. These data are consistent with the thesis that nitrite is bioactivated during hypoxic stress and consistent with recent studies of Bryan and colleagues demonstrating an acute conversion of tissue nitrite to RSNO and RxNO after a systemic anoxic insult (*Proc Natl Acad Sci U S A.*, 2004). The low levels of nitrite that are cytoprotective (1.2 nmoles at lowest dose – Figure 6B and 6C) and the reductive decomposition of "native" liver nitrite in the saline treated control animals (Figure 8A) suggest that this may be a natural mechanism for hypoxic NO production and cytoprotection. Consistent with the near-physiological amounts of nitrite given, blood nitrite levels were not significantly elevated (594 ± 83 nM to 727 ± 40 nM; n=3; p=0.16) in mice treated with 48 nmoles of nitrite, the most effective dose.

Cytoprotective effects of Nitrite are NO dependent, NO synthase Independent and Heme Oxygenase Independent: Further supporting a mechanism involving the hypoxic reduction of nitrite to NO, the NO inhibitor PTIO completely inhibited protective effects of nitrite in full factorial design experiments (Figure 9A). In contrast, significant nitrite cytoprotection was observed in endothelial NO synthase (eNOS) deficient mice (Figure 9B; $p < 0.001$), suggesting that NO production from nitrite during ischemia-reperfusion is eNOS independent. While heme oxygenase 1 protein expression is significantly induced following ischemia-reperfusion in this model, and appears to confer protection (Figure 9C and 9D), in mice pre-treated with ZnDPBG (a specific and potent heme oxygenase 1 inhibitor) nitrite significantly limited tissue injury suggesting a heme oxygenase-independent effect (Figure 9C; $p < 0.05$).

Discussion

In this example, nitrite treatment significantly increased the levels of liver nitrite and nitros(yl)ated species (RSNO and RXNO), compared with saline and nitrate treated controls, and conferred a dramatic dose-dependent cytoprotective effect, limiting necrosis, apoptosis, and preserving organ function. Remarkably, the levels of nitrite added were near-physiological, with a protective effect observed at even 1.2 nmoles added nitrite (a calculated blood level of 600 nM), suggesting that this may represent an endogenous protective mechanism that buffers severe metabolic or pathophysiological stress.

Recent data suggest that nitrite concentrations vary between blood and different organs and are typically in the high nanomolar to low micromolar range. However, until recently the high concentrations required to vasodilate aortic ring preparations led to its dismissal as an important biologically active molecule. Indeed, Furchgott *et al.* (*J. Pharmaco. Exper. Thera.* 108:129-143, 1953) demonstrated in 1953 that 100 μM nitrite stimulated vasodilation of aortic ring preparations, a process later shown to be mediated by activation of soluble guanylate cyclase (Kimura *et al., J Biol*

Chem 250:8016-8022, 1975; Mittal *et al.*, *J Biol Chem* 253:1266-1271, 1978; Ignarro *et al.*, *Biochim Biophys Acta* 631:221-231, 1980; Ignarro *et al.*, *J Pharmacol Exp Ther* 218:739-749, 1981). From a physiological standpoint, the *in vivo* conversion of nitrite to NO was thought to be limited to the stomach and severely ischemic heart, where acidic reduction or disproportionation at very low pH produces gastric mucosal vasodilation (Gladwin *et al.*, *J Clin Invest* 113:19-21, 2004; Bjorne *et al.*, *J Clin Invest* 113:106-114, 2004) and apparent cardiac tissue injury and heme iron-nitrosylation (at high nitrite concentrations in ischemic *ex vivo* heart preparations; Tiravanti *et al.*, *J Biol Chem* 279:11065-11073, 2004), respectively. While xanthine oxidoreductase dependent nitrite reduction can occur at very low oxygen tensions, NO production from this system is only detectable in the presence of high concentrations of superoxide dismutase (Li *et al.*, *J Biol Chem* 279:16939-16946, 2004; Li *et al.*, *Biochemistry* 42:1150-1159, 2001).

As described in Figure 6 and Cosby *et al.* (*Nat Med* 9:1498-1505, 2003), infusions of sodium nitrite into the human circulation produced significant vasodilation at both pharmacological and near-physiological concentrations. The bioactivation of nitrite appeared to be mediated by a nitrite reductase activity of deoxygenated hemoglobin, ultimately forming NO and iron-nitrosylated hemoglobin, and to a lesser extent S-nitrosated protein species. Based on these data, a role for circulating nitrite in mediating hypoxic vasodilation was proposed, with the oxygen sensor in this case being hemoglobin (Cosby *et al.*, *Nat Med* 9:1498-1505, 2003). It is now proposed that a similar nitrite reductase activity of deoxyhemoglobin, deoxymyoglobin and/or other deoxygenated heme proteins, accounts for the formation of nitros(yl)ated proteins and apparent NO-dependent cytoprotection observed during liver and cardiac ischemia in the present example.

Though the precise mechanism of how nitrite confers tissue protection is unclear, a critical role for NO is implicated from data shown in Figure 3 and 9A. Previous studies of NO and ischemia-reperfusion have yielded conflicting reports regarding the effects of NO on the severity of I/R injury, with some studies suggesting that NO actually contributed to reperfusion injury (Woolfson *et al.*, *Circulation* 91:1545-1551, 1995; Wink *et al.*, *Am J Physiol Heart Circ Physiol* 285:H2264-2276, 2003). Our laboratory has previously demonstrated that NO donors as well as the NO precursor, L-arginine, protect against myocardial I/R injury (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Nakanishi *et al.*, *Am J Physiol* 263:H1650-1658, 1992; Pabla *et al.*, *Am J Physiol* 269:H1113-1121, 1995). More recently, we demonstrated that the severity of myocardial I/R injury is markedly exacerbated in eNOS-/- mice (Jones *et al.*, *Am J Physiol* 276:H1567-1573, 1999) whereas mice with eNOS overexpression are protected against myocardial infarction and subsequent congestive heart failure (Jones *et al.*, *Am J Physiol Heart Circ Physiol* 286:H276-282, 2004; Jones *et al.*, *Proc Natl Acad Sci U S A* 100:4891-4896. 2003; Jones *et al.*, *Am J Physiol* 276:H1567-1573, 1999).

Conflicting data on the effects of NO on ischemia-reperfusion injury may be related to the dose of NO and the conditions during ischemia and reperfusion (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001). It is now well appreciated that very high, non-physiological levels of NO (*i.e.*, high micromolar and millimolar) actually promote cellular necrosis and apoptosis (Dimmeler *et al.*, *Nitric Oxide* 4:275-281, 1997), while the demonstrated cytoprotective effects of NO typically involve

nanomolar or low micromolar concentrations of NO (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993; Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001). Additionally, studies investigating NO and NO-releasing agents under *in vitro* conditions of I/R have consistently reported deleterious effects of NO (Bolli, *J. Mol. Cell. Cardio.* 33:1897-1918, 2001), in contrast to *in vivo* studies of I/R that reported beneficial effects of NO therapy (Lefer *et al.*, *New Horiz* 3:105-112, 1995; Lefer *et al.*, *Circulation* 88:2337-2350, 1993). How NO mediates protection is also not clear, with multiple mechanisms being reported, including sGC activation, inhibition of cytochrome C oxidase and inhibition of deleterious mitochondrial calcium uptake (Torres *et al.*, *FEBS Lett* 475:263-266, 2000; Brown *et al.*, *FEBS Lett* 356:295-298, 1994; Cleeter *et al.*, *FEBS Lett* 345:50-54, 1994; Rakhit *et al.*, *Circulation* 103:2617-2623, 2001). While these data suggest that the effects of nitrite occur secondary to NO formation, the ultimate mechanism of nitrite-dependent cytoprotection is currently unknown (Luchsinger *et al.*, *Proc Natl Acad Sci US A* 100:461-466, 2003; Fernandez *et al.*, *Inorg Chem* 42:2-4, 2003; Han *et al.*, *Proc Natl Acad Sci U S A* 99:7763-7768, 2002; Crawford *et al.*, *Blood* 101:4408-4415, 2003).

An intriguing possibility is the intermediate formation of S-nitrosothiols, known to form via reactions of nitrite with deoxyhemoglobin and possibly tissue heme proteins (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Cosby *et al.*, *Nat Med* 9:1498-1505, 2003; Nagababu *et al.*, *J Biol Chem* 278:46349-46356, 2003). Consistent with hypoxia dependent formation of S-nitrosothiols in red blood cells and tissues from nitrite, hepatic levels of these species were significantly higher following reperfusion (one-to-thirty minutes) in livers exposed to ischemia and nitrite. Within the relative reductive environment intracellularly, S-nitrosothiols formed via nitrite readily will be reduced to NO and activate sGC. Alternatively, S-nitrosation and subsequent effects on activity of critical proteins important in I/R induced injury and apoptotic cell death may lead to protection (Mannick *et al.*, *Science* 284:651-654, 1999).

In addition, the data reported here reveal a dynamic regulation of hepatic RxNO's, a pool of mercury stable NO-modified proteins that include N-nitrosamines and iron-nitrosyls (Bryan *et al.*, *Proc Natl Acad Sci U S A.*, 2004; Gladwin *et al.*, *J Biol Chem* 21:21, 2002; Rassaf *et al.*, *Free Radic Biol Med* 33:1590-1596, 2002), during ischemia-reperfusion. In saline treated groups, RxNO levels increase at 1 minutes of reperfusion and then decrease after 30 minutes reperfusion, whereas sustained elevation in RxNO levels are observed in nitrite treated mice, suggesting that maintenance of RxNO's could be important in protecting tissues from I/R injury.

In conclusion, the data presented in this example demonstrate a remarkable function for the relatively simple inorganic anion nitrite as a potent inhibitor of liver and cardiac ischemia-reperfusion injury and infarction, as shown in a mouse model system. The effects of nitrite appear NO-dependent, with a rapid conversion of nitrite to NO and nitros(yl)ated proteins following reperfusion. Considering the known safety of nitrite as a naturally occurring anion and as an FDA approved therapeutic for cyanide poisoning, these data evince a novel, safe, and inexpensive therapy for ischemia-reperfusion injury. Such a therapy could be used to prevent or modulate organ dysfunction following, for instance, coronary and peripheral vasculature reperfusion, high risk abdominal surgery

(such as aortic aneurism repair that leads to renal acute tubular necrosis), cardiopulmonary resuscitation, and perhaps most importantly, solid organ transplantation.

Example 3

Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator

This example provides a description of use of inhaled, nebulized nitrite (specifically, sodium nitrite) to treat neonatal pulmonary hypertension.

Based on the results presented above, it is now known that the blood anion nitrite contributes to hypoxic vasodilation via a heme-based, nitric oxide (NO) generating reaction with deoxyhemoglobin and potentially other heme proteins. This biochemical reaction can be harnessed for the treatment of neonatal pulmonary hypertension, an NO-deficient state characterized by pulmonary vasoconstriction, right-to-left shunt pathophysiology, ventilation/perfusion inhomogeneity and systemic hypoxemia. As shown in this example, inhaled sodium nitrite was delivered by aerosol to newborn lambs with hypoxic and normoxic pulmonary hypertension. Inhaled nitrite elicited a rapid and sustained reduction (~60%) in hypoxia induced pulmonary hypertension, a magnitude approaching that of the effects of 20 ppm NO gas inhalation and which was associated with the immediate appearance of increasing levels of NO in expiratory gas. Pulmonary vasodilation elicited by aerosolized nitrite was deoxyhemoglobin- and pH-dependent and was associated with increased blood levels of hemoglobin iron-nitrosylation. Significantly, from a therapeutic standpoint, short term delivery of nitrite, dissolved in saline, via nebulization produced selective and sustained pulmonary vasodilation with no appreciable increase in blood methemoglobin levels. These data support the paradigm that nitrite is a vasodilator acting via conversion to NO, a process coupled to hemoglobin deoxygenation and protonation, and further evince a novel, simple and inexpensive therapy for neonatal pulmonary hypertension.

The effect of nebulized sodium nitrite versus saline, or inhaled NO, on both hypoxia-induced and drug-induced pulmonary hypertension was compared in newborn lambs. As described in this example, inhaled nitrite forms expired NO gas and circulating iron-nitrosyl-hemoglobin, and selectively vasodilates the pulmonary circulation. This vasoactivity is associated with the level of hemoglobin desaturation and blood pH in the physiologic range, supporting the physiological and therapeutic paradigm of hemoglobin as a deoxygenation-linked nitrite reductase.

Methods

Animal protocols were approved by the Institutional Animal Research Committee of Loma Linda University and were in accordance with the National Institutes of Health guidelines for use of experimental animals.

Animal preparation: Following induction of anesthesia with intravenous thiopental sodium (20 mg/Kg), the newborn lambs were orotracheally intubated and anesthesia maintained with 1% halothane until catheters were placed surgically. Thereafter halothane was discontinued and

anesthesia maintained with morphine (0.1 mg/kg/hr). After paralysis with vecuronium (0.1 mg/kg/hr) the lungs were mechanically ventilated with initial settings of pressures: 22/6 cm H_2O, frequency: 25 breaths per minute, FiO_2: 0.21, and inspiratory time: 0.6 seconds (Sechrist Model 100, Sechrist Industries, Anaheim CA, USA). Initially and throughout the normoxic experiments, ventilator settings of frequency, peak inspiratory pressure, and FiO_2 were adjusted to maintain $SaO_2 > 95\%$, PaO_2 at 90-150 Torr, and $PaCO_2$ at 35-45 Torr.

A catheter was placed in the right brachial artery to sample pre-ductal blood for gases and chemical analysis. A pediatric thermodilution catheter was passed through a femoral vein to the pulmonary artery to measure cardiac output, pulmonary artery and pulmonary capillary wedge pressure (5.0 Pediatric Swan-Ganz® thermodilution catheter, Baxter Healthcare Corporation, Irvine, CA, USA).

Catheters were placed in the femoral artery and vein for monitoring blood pressure, heart rate, and for administration of fluids and drugs. A thermocouple was placed in the femoral vein to monitor core-body temperature which was maintained at 39 C by using a warming blanket and heat lamp throughout the experiments.

After completion of the experiments, the lambs were euthanized with a proprietary euthanasia solution (Euthasol, Western Medical Supply, Arcadia, CA, USA). In selected experiments necropsy was performed to verify the position of catheters (which were correctly positioned in all cases) and to determine that the ductus arteriosus was closed (which was closed in all cases).

Hemodynamic measurements: Mean arterial pressure, mean pulmonary artery pressure, and central venous pressure were measured continuously, and pulmonary capillary wedge pressure was measured intermittently by using calibrated pressure transducers (COBE Laboratories, Lakewood, CO) zeroed at the midthoracic level. Cardiac output was measured at 15-minute intervals throughout the studies by thermodilution using a Com-2 thermodilution module (Baxter Medical, Irvine, CA, USA). Five-ml injections of ice-cold saline were used. Determinations were carried out in triplicate and results were averaged for each sampling time point. Pulmonary vascular resistance and systemic vascular resistance were calculated by using standard formulas.

Blood gas and methemoglobin analysis: Arterial and mixed venous pH, PCO_2, and PO_2 were measured in blood samples (0.3 ml) collected at intervals throughout the experiments. Blood gases were measured (ABL3, Radiometer, Copenhagen, Denmark) and oxyhemoglobin saturation and hemoglobin concentration were measured using a hemoximeter (OSM2 Hemoximeter, Radiometer, Copenhagen, Denmark). Arterial and mixed venous methemoglobin concentrations were analyzed by photometry with the OSM2 Hemoximeter using the same arterial sample as in the blood gas determinations.

Delivery of aerosolized nitrite, saline, or NO gas: Five milliliters of either aqueous sodium nitrite (1 mM solution) or saline were placed in a jet nebulizer (Hudson RCI Micro Mist Nebulizer (Hudson Respiratory Care; Temucula, CA), driven at a constant flow rate of 8 L/minute in all experiments. The sodium nitrite solution was nebulized at a rate of 270 µmol/minute. Aerosols were delivered to the inspiration loop of the ventilator. Using a jet nebulizer, it is generally thought

that <10% of a nebulized drug deposits in the lung (Coates *et al.*, *Chest* 119, 1123-30, 2001). This is the result of the dead volume of the nebulizer and the loss of drug during the expiratory phase. Lung deposition depends on particle size distribution, which is under the influence of air flow, filling volume, drug solution, and ambient temperature (Flavin *et al.*, *Pediatr Pulmonol* 2, 35-9, 1986; Suarez & Hickey, *Respir Care* 45, 652-66, 2000; Clay *et al.*, *Thorax* 38, 755-9, 1983; Clay *et al.*, *Lancet* 2, 592-4, 1983). This is a simple, inexpensive, and widely available clinical nebulizer system, though other systems could be used.

NO gas was introduced into the inspiratory limb of the breathing circuit. The inspired concentration of NO was continuously measured by chemiluminescence (CLD 700 AL, Eco Physics Inc, Ann Arbor, MI) in the inspiratory limb of the ventilator loop.

Inhalation of nitrite or saline aerosols during hypoxic- induced pulmonary vasoconstriction. Seven lambs were studied in order to demonstrate that nebulized nitrite is a selective pulmonary vasodilator in hypoxic newborn lambs. After anesthesia and instrumentation, the lambs were allowed to recover for 30 to 90 minutes while relevant hemodynamic parameters were monitored. After baseline measurements were obtained, a 30-minute period of pulmonary hypertension was induced by decreasing the FiO_2 of the inspired gas to 0.12 for 30 minutes. Ten minutes after initiation of hypoxia, either saline or sodium nitrite aerosols were administered for the remainder of the hypoxic period. After a one-hour recovery period, a second 30-minute period of hypoxia was induced again with either saline or sodium nitrite aerosols administered during the last 20 minutes. Arterial blood samples for blood gases and analytical assays were drawn and cardiac output measurements were performed at regular intervals.

Inhalation of nitrite during U46619-induced pulmonary hypertension in normoxic conditions. Six additional lambs were studied in order to evaluate the effects of nitrite nebulization on normoxic pulmonary hypertension. Stable normoxic pulmonary hypertension was induced by an infusion of a stable endoperoxide analog of thromboxane (U46619 - 9, 11-dideoxy-11α-epoxymethano-prostaglandin $F_{2\alpha}$, Cayman Chemicals, Ann Arbor, MI). The drug was dissolved in saline and was administered at a rate of 2 μg/kg/min into the femoral venous catheter for 30 minutes. Nitrite was nebulized for inhalation during the last 20 minutes of the infusion (Figure 11).

Comparison of inhaled nitrite and NO gas during hypoxic-induced pulmonary vasoconstriction: efficacy and duration of effect. This protocol was designed to compare the efficacy of nitrite with the clinical standard, 20 ppm inhaled NO gas. This concentration of NO gas is at the upper end of the therapeutic dose given to infants with primary pulmonary hypertension (Kinsella & Abman, *Semin Perinatol* 24, 387-95, 2000; Kinsella *et al.*, *Lancet* 340, 819-20, 1992), and has also been shown to be effective in reversing hypoxic vasoconstriction in newborn lambs (Frostell *et al.*, *Circulation* 83, 2038-47, 1991). A second purpose was to determine the duration of effect of a short nitrite nebulization versus NO gas inhalation on hemodynamic and physiological measurements during prolonged hypoxic-induced pulmonary vasoconstriction. After baseline measurements were performed, the lambs were made hypoxic as described above for 35 minutes.

Ten minutes after initiation of hypoxia, a 20-minute period of NO gas inhalation was initiated (20 ppm), with continuation of hypoxia for 5 minutes after cessation of NO gas delivery. Lambs were then allowed to recover for one hour. Again, after baseline measurements were made, a second 90-minute period of hypoxia was initiated. Ten minutes after initiation of hypoxia, sodium nitrite aerosol was administered for 20 minutes, with continuation of hypoxia for 60 minutes after cessation of nitrite aerosolization (Figure 13).

Measurement of exhaled NO. Exhaled NO concentration was measured with a chemiluminescence NO analyzer (NOA 280, Sievers Instruments, Inc., Boulder, CO). The chemiluminescence analyzer was calibrated with NO-free air and NO gas (45 parts per million) according to the manufacturer's recommendations. NO was sampled though a Teflon sidearm attached to a sampling port at the proximal end of the endotracheal tube through which flow passed to the analyzer at 250 ml/min.

In selected early experiments, nitrite was nebulized through a ventilator circuit with no lamb connected while NO was measured with the chemiluminescence NO analyzer. In no experiments did nitrite nebulization through the disconnected circuit result in an increase in NO concentration in the ventilated air.

Measurement of plasma nitrite and iron-nitrosyl-hemoglobin. Blood was drawn from both the brachial artery and central venous catheter and rapidly processed. Plasma was separated after centrifugation, frozen immediately on dry ice, and then stored at –70 C until assayed for nitrite using the chemiluminescence methodologies (Sievers model 280 NO-analyzer) as previously described (Cosby *et al.*, *Nat Med* 9, 1498-505, 2003; Gladwin *et al.*, *J Biol Chem* 277, 27818-28, 2002; Yang *et al.*, *Free Radic Res* 37, 1-10, 2003). The frozen red blood cell pellet was thawed, reacted in 8 mM NEM, 100 μM DTPA, and 4 mM ferricyanide, incubated for 5 minutes, and passed through a Sephadex G25 column (Yang *et al.*, *Free Radic Res* 37, 1-10, 2003; Xu *et al.*, *Proc Natl Acad Sci U S A* 100, 11303-8, 2003). The hemoglobin fraction from the G25 column was quantified by the method of Drabkin (*J. Biol. Chem.* 112, 51-65, 1935)and reacted in 0.1 M HCl/0.5%sulfanilamide to eliminate residual nitrite. The samples were then injected into a solution of tri-iodide (I_3^-) in-line with a chemiluminescent nitric oxide analyzer (Sievers, Model 280 NO analyzer, Boulder, CO). NO gas is striped in the tri-iodide solution stoichiometrically from iron-nitrosyl-hemoglobin (Yang *et al.*, *Free Radic Res* 37, 1-10, 2003).

Electron paramagnetic resonance spectroscopy of whole blood. This was carried out at 110K using a Bruker 4131VT temperature controller on an EMX 10/12 EPR spectrometer system set at 9.4 GHz, 10 mW, 5 G modulation, 0.08 s time constant, and 84 s scan time over 600 G. Each curve represents a single 84-second scan. Concentrations of iron-nitrosyl-hemoglobin were calculated by comparing the peak-to-peak heights to a standard sample.

Data acquisition and analysis. Mean arterial pressure, pulmonary artery pressure, central venous pressure, heart rate, exhaled NO concentration, and core body temperature were measured continuously. Analog signals were digitized at 100 Hz and stored using an analogue-to-digital

converter (PowerLab SP, ADInstruments, Colorado Springs, CO) and data acquisition software (Chart v 5.02 for Macintosh, ADInstruments, Colorado Springs, CO). Following the experiments, arterial blood pressure, central venous pressure, heart rate, and exhaled NO measurements were averaged into 60-second blocks.

Statistical analysis. Serial measurements of physiological variables were compared by two-way ANOVA with repeated measures with group and time as the factors. Significance of differences was evaluated with a Dunnett's post-test. Significant differences from the baseline period were evaluated using one-way-ANOVA with repeated measures with individual animals and time as the factors. Significance of differences was further evaluated with a Newman-Keul's post-test. The calculations were done using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was assumed with $P < 0.05$. Data are presented as mean ± SEM.

Results

Pulmonary vasodilatory properties of aerosolized nitrite during hypoxic-induced pulmonary vasoconstriction

In order to determine the effect of nebulized nitrite on hypoxic pulmonary hypertension, seven newborn lambs (2-10 days of age) were instrumented under general anesthesia and maintained on mechanical ventilators and morphine infusion. Following baseline stabilization, the lambs were subjected to a 30-minute period of hypoxia by lowering FiO_2 to 0.12. Nebulized nitrite or saline was administered for the last 20 minutes of the hypoxic period. Initiation of hypoxia (arterial HbO_2 ~55%) was associated with rapid increases in mean pulmonary artery pressure (from 21 ± 1 to 34 ± 2 mmHg, $P < 0.01$) (Figure 10A, 10B) and pulmonary vascular resistance (20% ($P < 0.01$)), and decreased systemic vascular resistance (~20% ($P < 0.01$)). Inhalation of nebulized nitrite but not saline (Figure 10A, 10B) resulted in a selective decrease in pulmonary artery pressure by ~60% ($P < 0.01$) (Figure 10A, 10C) and reduced pulmonary artery resistance by ~70% ($P < 0.05$) but had no measurable effect on mean arterial blood pressure (Figure 10A, 10C) or systemic vascular resistance when compared to control animals. The decrease in pulmonary artery pressure with nitrite nebulization was associated with a progressive increase in exhaled NO from 3 ± 1 to 15 ± 4 ppb (Figure 10A, 10C). Cardiac output, arterial oxyhemoglobin saturation, and methemoglobin levels did not change measurably after nitrite inhalation as compared to values during the preceding ten minutes of hypoxia (Figure 10A). Arterial P_{O_2} could not change appreciably in our system as this was experimentally clamped.

Pulmonary vasodilating properties of aerosolized nitrite during normoxic drug-induced pulmonary vasoconstriction

In order to contrast the effects of nebulized nitrite on pulmonary artery pressure in the presence of normal deoxyhemoglobin with those in the presence of reduced oxygenated hemoglobin, the effects of nebulized nitrite were studied in a separate group of six lambs subjected to pulmonary hypertension under normoxic conditions. Stable normoxic (SaO_2 ~98%) pulmonary hypertension

was induced by infusion of the endoperoxide analog of thromboxane (U46619). Intravenous infusion of U46619 at a rate of 2 μg/kg/min for 30 minutes was associated with rapid increases in pulmonary artery pressure from 24 ± 1 to 51 ± 4 mmHg ($P < 0.001$) (Figure 11). Ten minutes after the infusion began, addition of inhalation of nebulized nitrite resulted in a selective decrease in pulmonary artery pressure by 23 ± 6% ($P < 0.05$ compared to infusion baseline), but had no effect on mean arterial blood pressure or systemic vascular resistance (Figure 11). The decrease in pulmonary artery pressure with nitrite nebulization was associated with a progressive increase in exhaled NO from 4.8 ± 1.2 to 10.1 ± 2.0 ppb ($P < 0.05$ compared to baseline, Figure 11). Figure 2 shows a comparison of the effects of nitrite inhalation after 20 minutes on hypoxic versus drug-induced normoxic pulmonary vasoconstriction. The changes in mean pulmonary artery pressure and exhaled NO were significantly larger with nitrite treatment during hypoxic conditions. Overall the effects of nitrite inhalation on normoxic (thromboxane-induced) pulmonary hypertension were less than those observed with hypoxic pulmonary hypertension (Figures 10, 11, 12A), consistent with a model of hypoxemic and possibly acidemic potentiation of nitrite's vasoactivity.

pH and oxygen dependence of the nitrite reductase activity of deoxyhemoglobin

We hypothesize that the biochemical conversion of nitrite to NO requires both deoxyhemoglobin and protonation. Thus, data from both the normoxic and hypoxic experiments were used to study the influence of hemoglobin saturation and pH on NO production from nitrite. Measurements of exhaled NO gas and NO-modified hemoglobin (iron-nitrosyl-hemoglobin) were used as both dosimeters of NO production and as a measure of the direct byproducts of the nitrite reductase reaction of nitrite and hemoglobin to produce NO. Figure 12 shows that iron-nitrosyl-hemoglobin, measured by tri-iodide based reductive chemiluminescence (Figure 12B) and electron paramagnetic resonance (Figure 12C), was markedly increased by nitrite inhalation during hypoxia but not with drug-induced normoxic pulmonary vasoconstriction. As shown in Figure 12D, change in mean pulmonary artery pressure during hypoxia after inhalation of nebulized sodium nitrite was related to blood pH, with increased vasodilation associated with decreasing pH ($r = 0.57$ $P = 0.055$).

Comparison with inhaled NO and duration of effect.

We next compared the efficacy of nitrite with the current therapeutic standard, inhaled NO gas. After initiation of hypoxia, lambs were subjected to (20 ppm) inhaled NO gas or nebulized nitrite for 20 minutes. The data in Figure 13 show the duration and magnitude of the effect of NO gas inhalation (Figure 13A) or nitrite nebulization (Figure 13B, 13C) on hemodynamic and metabolic measurements during hypoxia. Although both treatments resulted in a pronounced reduction in hypoxic pulmonary hypertension, the response to inhaled NO gas was slightly more rapid and pulmonary pressure more nearly approached baseline when contrasted to the 60-70% correction in pressure elicited by nitrite. Systemically, mean arterial blood pressure and resistance was reduced to a similar extent with both treatments during hypoxia. However, with the relative chemical stability of the nitrite anion compared with NO gas, there was sustained vasodilation for more than 60 minutes

(the duration of the hypoxic challenge) after discontinuation of nitrite inhalation, whereas the termination of NO gas delivery abolished the vasodilating effect in a matter of seconds (Figure 13A, 13B). The relatively sustained effect of nitrite nebulization might be therapeutically advantageous by allowing for intermittent therapy analogous to the treatment of asthma with beta-adrenergic agonists by meter dose inhaler. The time course of nitrite inhalation-induced pulmonary vasodilation and plasma nitrite levels are shown (Figure 13C, 13D). In this experiment which tracked biochemical changes for a longer period that in Figure 10 methemoglobin (MetHb) concentrations increased from 2.1 ± 0.1 % during baseline to 2.8 ± 0.2% after nitrite nebulization ($P < 0.05$).

Discussion

A principle finding of this example is that a brief period of inhalation of nebulized sodium nitrite solution produces rapid and selective pulmonary vasodilation during hypoxic-induced pulmonary hypertension in newborn lambs. The significant reduction in pulmonary artery pressure following nitrite nebulization was sustained when hypoxia was continued for more than an hour after termination of nitrite nebulization. In none of the experiments did nitrite inhalation produce systemic hypotension, and methemoglobin elevation was minimal. From a mechanistic standpoint, nitrite administration was associated with NO production, measured by exhaled NO gas and NO-modified hemoglobin, with responses in proportion to levels of hemoglobin-oxygen desaturation and decreases in blood pH. These data support the paradigm that nitrite is an NO-dependent vasodilator whose bioactivation is coupled to hemoglobin deoxygenation and protonation.

Inhaled NO gas is the current standard for the treatment of pulmonary hypertension. Figure 13 provides a comparison of the effects of NO gas at 20 ppm with those of aerosolized nitrite. In about 5 minutes the NO gas effectively ablated about 80% of hypoxic-induced pulmonary hypertension, an effect that was short lived but which could be reproduced when it was given again 20 minutes later. Aerosolized sodium nitrite removed about 60% of hypoxic-induced pulmonary hypertension. This response was consistently observed in each of the lambs studied and it persisted throughout the one-hour period of hypoxia that was maintained after the nitrite aerosol was discontinued. The changes in pulmonary blood flow were accompanied by corresponding changes in the calculated resistance to blood flow through the lungs, indicating that changes were in the pulmonary vasculature rather than secondary to changes in cardiac output or systemic effects that might have altered perfusion pressures.

We demonstrate herein that aerosolized nitrite is an NO producing agent in the newborn lamb that can be readily administered by nebulization and appears to exhibit a wide therapeutic-to-safety margin, with limited systemic hemodynamic changes and methemoglobin production. This presents an attractive therapeutic option to inhaled NO. Nitrite is an ideal "NO producing" agent in that it 1) is a naturally occurring compound in blood, alveolar lining fluid, and tissue, and 2) has no parent-compound leaving group, such as the diazenium diolates, that requires extensive toxicological study prior to translation to human disease, and 3) it is already approved for human use in cyanide antidote kits. These advantages are to be counterbalanced against possible problems that might occur

with more prolonged delivery, including alveolar nitrite accumulation, systemic vasodilation, and the development of methemoglobinemia.

In conclusion, the data presented in this example suggest that inhaled nitrite is a potent and selective vasodilator of pulmonary circulation of the newborn lamb and further support the paradigm that nitrite, and particularly salts of nitrite, such as sodium nitrite, is an NO-dependent vasodilator whose bioactivation is coupled to hemoglobin deoxygenation and protonation. In none of our studies did inhaling nitrite produce systemic hypotension or elevate methemoglobin levels.

Example 4

Use of nitrite infusions for the prevention of cerebral artery vasospasm after subarachnoid hemorrhage

This example describes a method for using nitrite infusion to prevent cerebral artery vasospasm after intracranial hemorrhage.

Subarachnoid hemorrhage (SAH) due to the rupture of intracranial aneurysms affects 28,000 Americans annually. Almost 70% of patients with aneurysmal SAH develop severe spasm of the cerebral arteries on the seventh day after SAH. Despite aggressive medical therapy, neurological deficits resulting from vasospasm continue to be a major cause of morbidity and mortality. Although the etiology of cerebral vasospasm is poorly understood, there is increasing evidence that erythrocyte hemolysis in the cerebrospinal fluid and decreased availability of nitric oxide (NO), a potent vasodilator, plays a significant role. Reversal of vasospasm by NO or NO prodrugs has been documented in several animal models.

Despite half a century of research and clinical trials, delayed cerebral vasospasm (DCV) remains the single cause of permanent neurological deficits or death in at least fifteen percent of patients following otherwise successful endovascular or surgical treatment for ruptured intracranial aneurysm. Decreased bioavailability of nitric oxide (NO) has been mechanistically associated with the development of DCV. This work was carried out to determine whether infusions of nitrite, a naturally occurring anion that reacts with deoxyhemoglobin to form NO and S-nitrosothiol, might prevent DCV via reactions with perivascular hemoglobin.

Methods

An autologous arterial blood clot was placed around the right middle cerebral artery (R MCA) of 14 anesthetized *Cynomolgus* monkeys at day 0. Sodium nitrite solution ($NaNO_2$, 135 mg/daily and 180 mg/daily, which approximates 45 mg/kg and 60 mg/kg per day) in 0.9% saline (n=6) or saline alone (n=8) was infused intravenously for 14 days in awake animals via an ambulatory MiniMed Infusion Pump, at 2μl/minute. Cerebral arteriogram was performed before clot placement and on days 7 and 14, for assessment of DCV. Arteriographic vasospasm was defined as a 25% or greater reduction in the proximal 14 mm of the R MCA area as measured on the AP projection of the cerebral arteriogram (blinded assessment). Mean arterial blood pressure was

measured and blood samples were collected daily from day 0; the cerebral spinal fluid samples were collected on day 0, 7, and 14.

Results

In control animals, cerebral spinal fluid nitrite levels decreased from 3.1±1.5 μM to 0.4±0.1 μM at 7 days and 0.4±0.4 μM at 14 days (Figure 14), and all eight animals developed significant vasospasm of the R MCA (Figures 15 and 16), complicated by stroke and death in one animal.

Nitrite infusions were associated with increases in plasma cerebrospinal fluid nitrite and blood methemoglobin concentrations without systemic hypotension (Figure 14), and significantly reduced the severity of vasospasm (Figures 15 and 16; no animals developed significant vasospasm; mean reduction in the R MCA area on day 7 after SAH was 8±9% versus 45±5%; $P < 0.001$). Pharmacological effects of nitrite infusion were associated with bioconversion of cerebrospinal fluid nitrite to S-nitrosothiol, a potent vasodilating NO donor intermediate of nitrite bioactivation. There was no clinical or pathological evidence of nitrite toxicity.

Conclusions

Subacute sodium nitrite infusions prevent DCV in a primate model of SAH, and do so without toxicity. These data evince a novel, safe, inexpensive, and rationally designed therapy for DCV, a disease for which no current preventative therapy exists.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments, and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

CLAIMS

1. A method for treating or ameliorating a condition selected from:

(a) hepatic or cardiac or brain ischemia-reperfusion injury;

(b) pulmonary hypertension; or

(c) cerebral artery vasospasm,

in a subject by decreasing blood pressure and/or increasing vasodilation in the subject, the method comprising administering sodium nitrite to the subject to decrease the blood pressure and/or increase vasodilation in the subject, thereby treating or ameliorating the condition.

2. The method of claim 1, which is a method for treating or ameliorating hepatic or cardiac or brain ischemia-reperfusion injury.

3. The method of claim 2, wherein administering sodium nitrite to the subject is intravenous.

4. The method of claim 2 or 3, wherein the sodium nitrite is administered to a circulating concentration of about 0.6 to 240 μM.

5. The method of claim 1, which is a method for treating or ameliorating pulmonary hypertension.

6. The method of claim 5, wherein the pulmonary hypertension is neonatal pulmonary hypertension.

7. The method of claim 5 or 6, wherein administering sodium nitrite to the subject is by inhalation.

8. The method of claim 7, wherein the sodium nitrite is nebulized.

9. The method of any one of claims 5 through 8, wherein the sodium nitrite is administered at a rate of 270 μmol/minute.

10. The method of claim 1, which is a method for treating or ameliorating cerebral artery vasospasm.

11. The method of claim 10, wherein administering sodium nitrite to the subject is intravenous.

12. The method of claim 10 or 11, wherein the sodium nitrite is administered at a rate of about 45 to 60 mg/kg.

13. The method of any one of claims 1-12, wherein the sodium nitrite is administered in combination with at least one additional agent.

14. The method of any one of claims 1-13, wherein the subject is a mammal.

15. The method of any one of claims 14, wherein the subject is a human.

Figure 1A

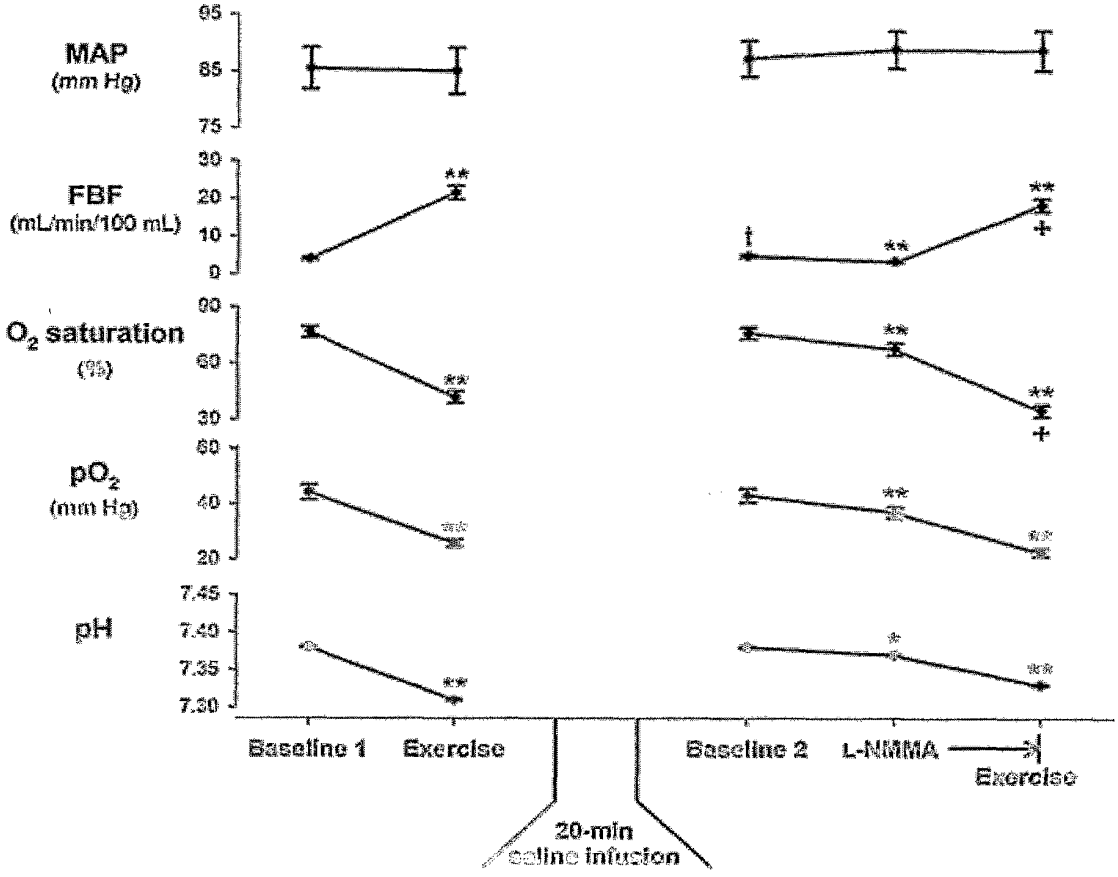


Figure 1B

Figure 2A

Figure 2B

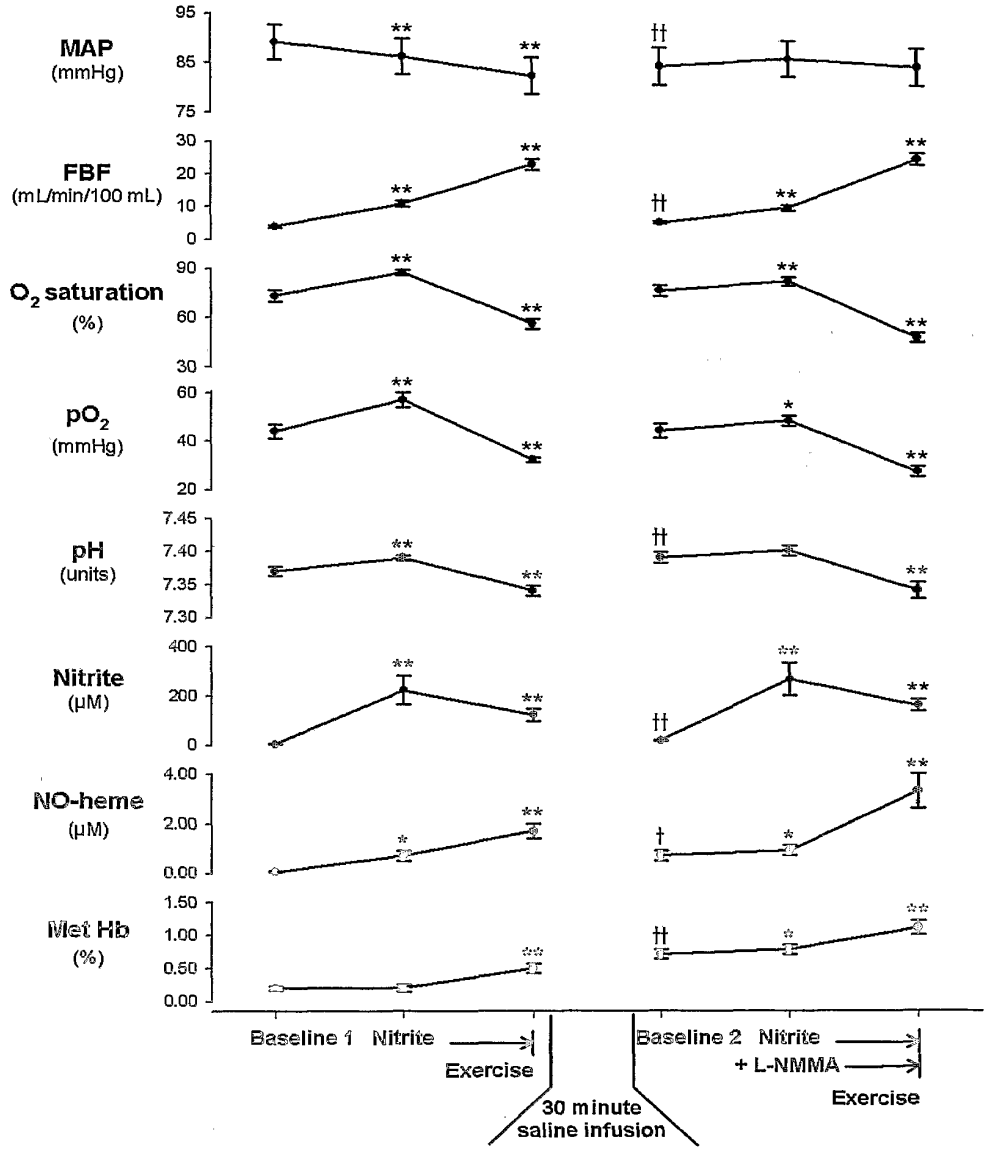


Figure 3A

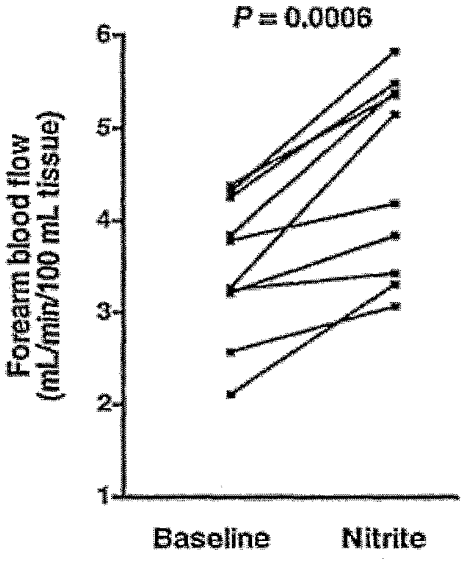


Figure 3B

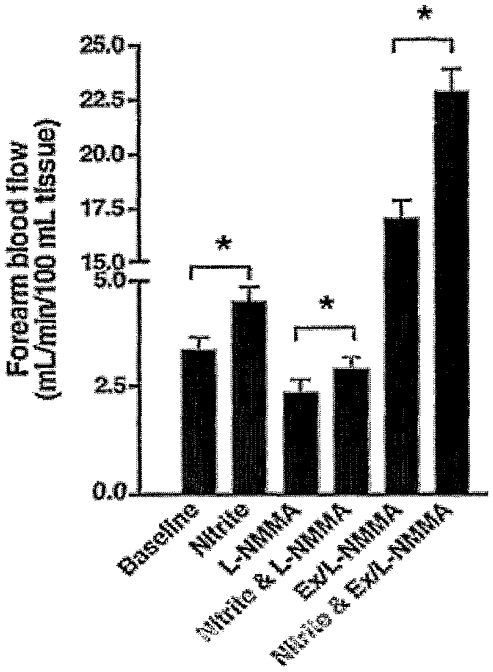


Figure 3C

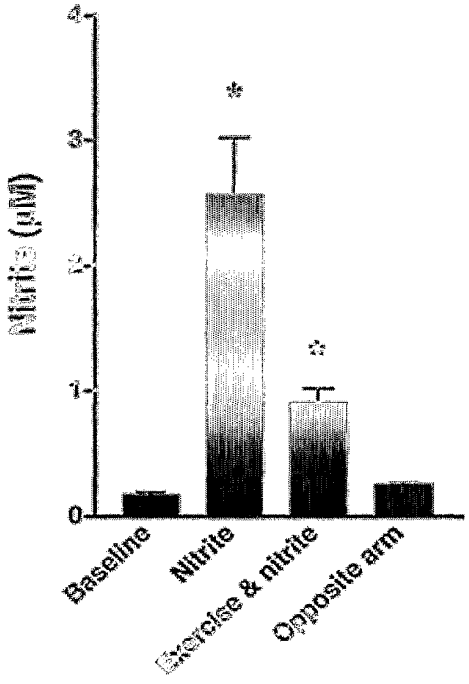


Figure 3D

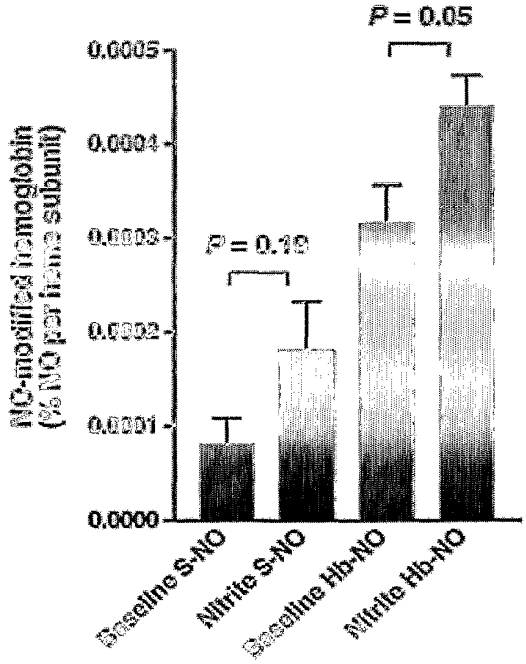


Figure 4A

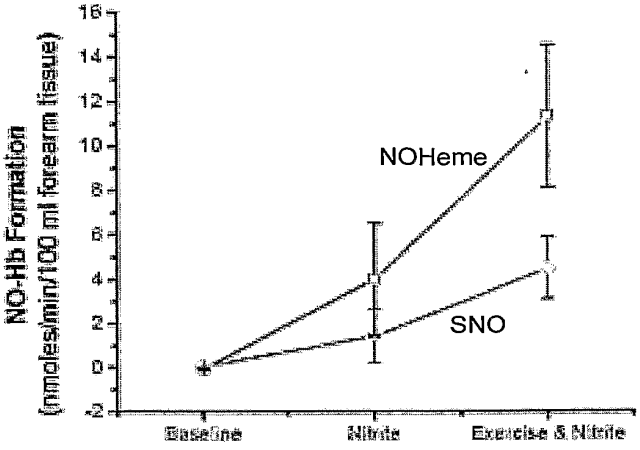


Figure 4B

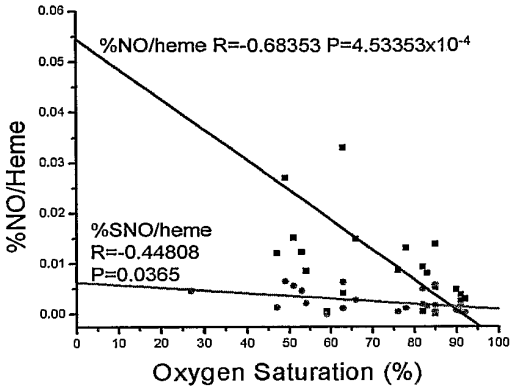


Figure 5A

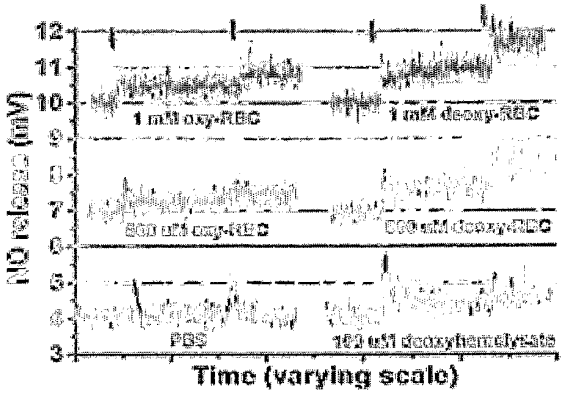


Figure 5B

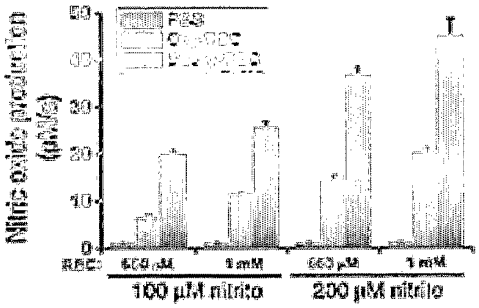


Figure 6A

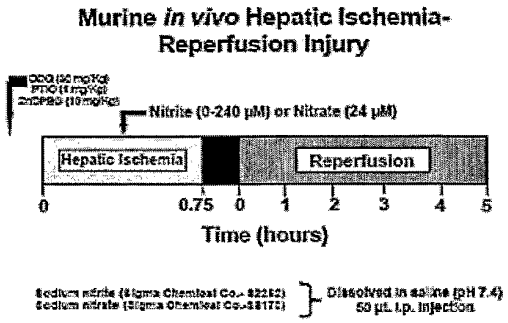


Figure 6B

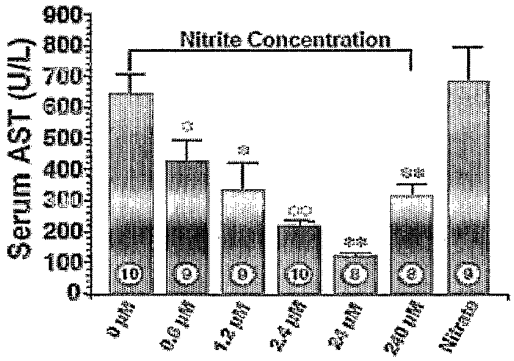


Figure 6C

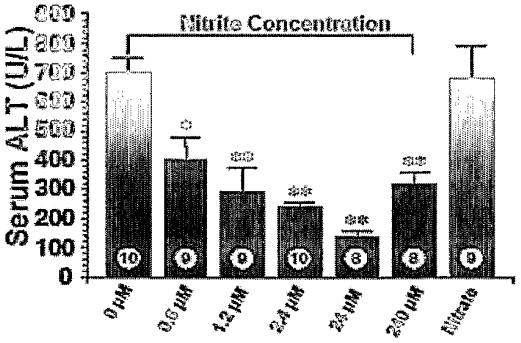


Figure 6D

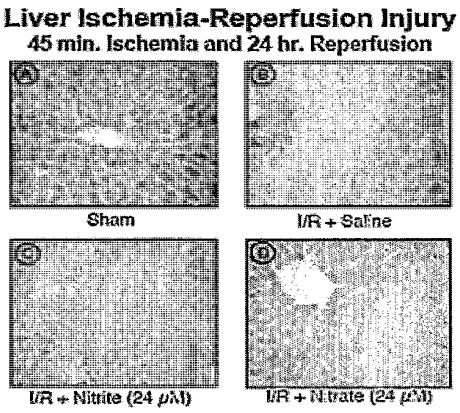


Figure 6E

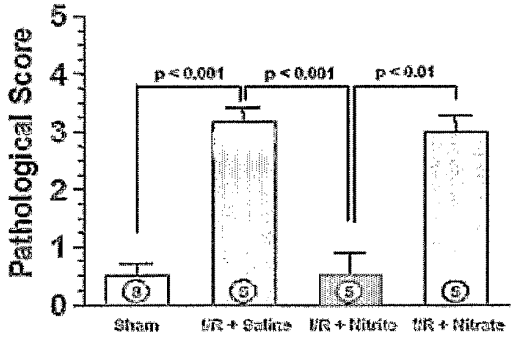


Figure 6F

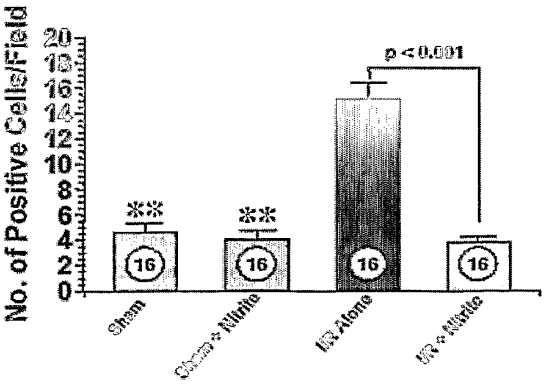


Figure 7A

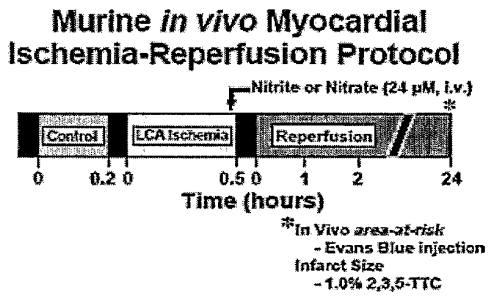


Figure 7B

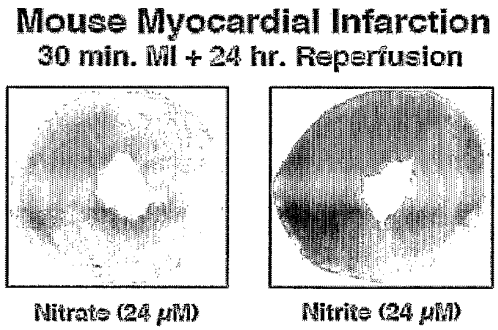


Figure 7C

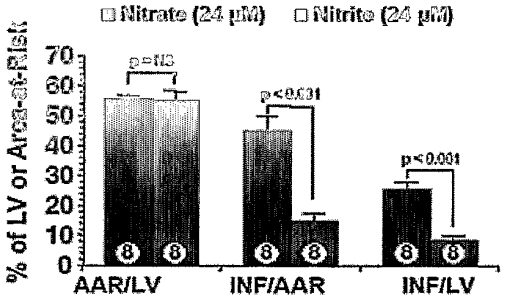
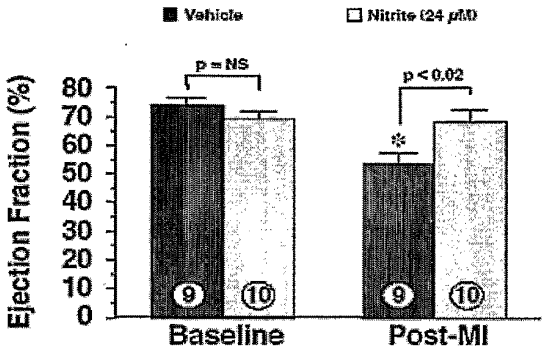


Figure 7D



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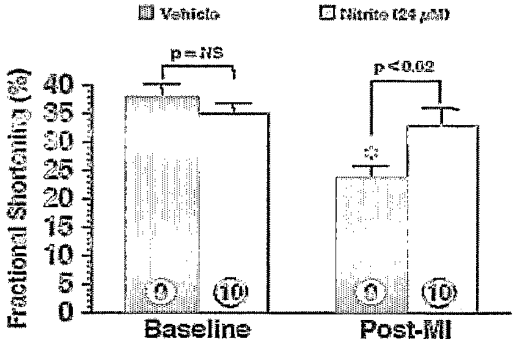
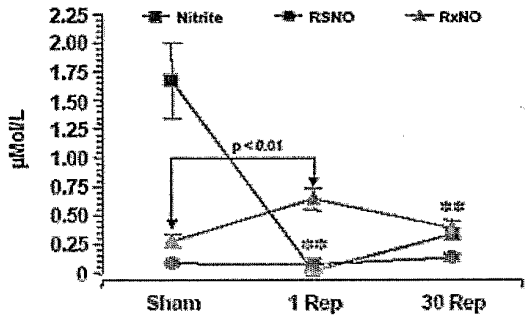


Figure 8A



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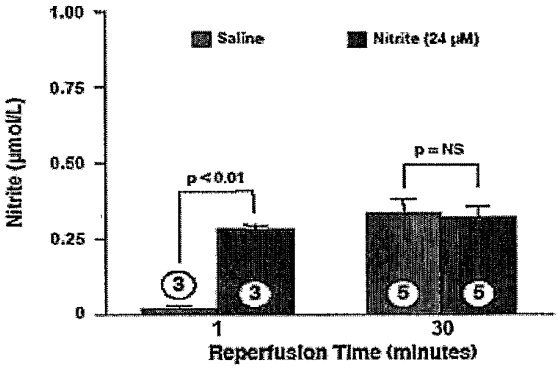


Figure 8C

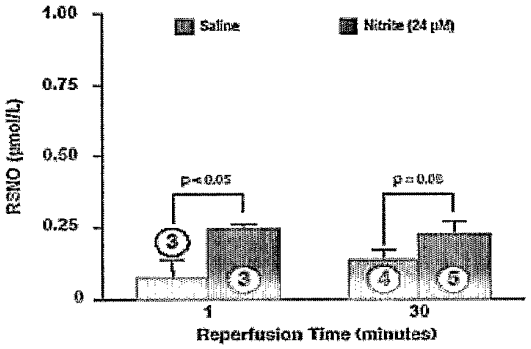


Figure 8D

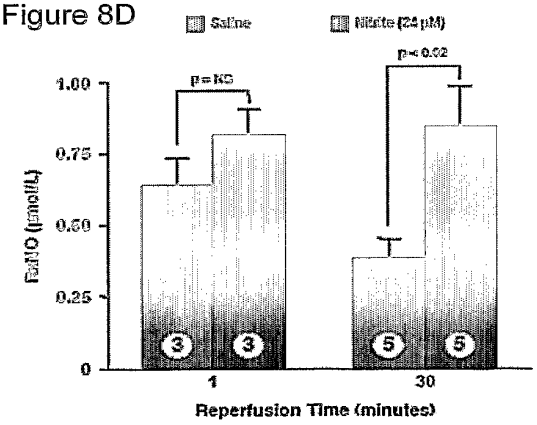


Figure 9A

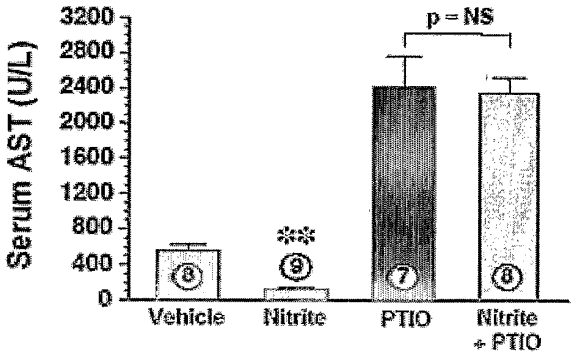


Figure 9B

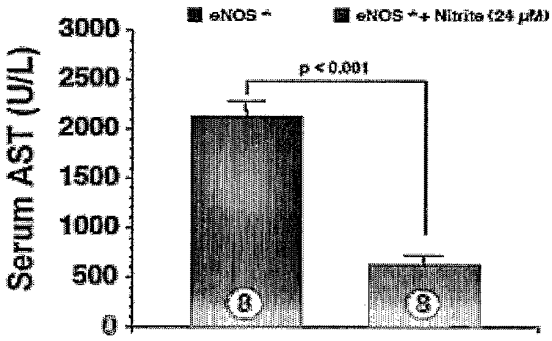


Figure 9C

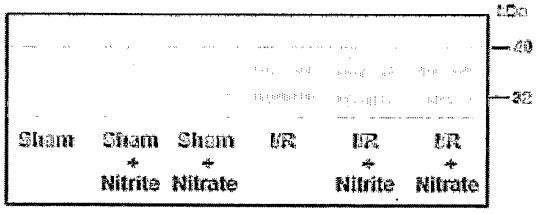


Figure 9D

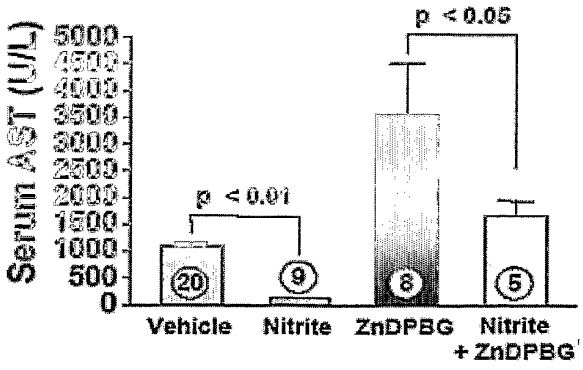


Figure 10A

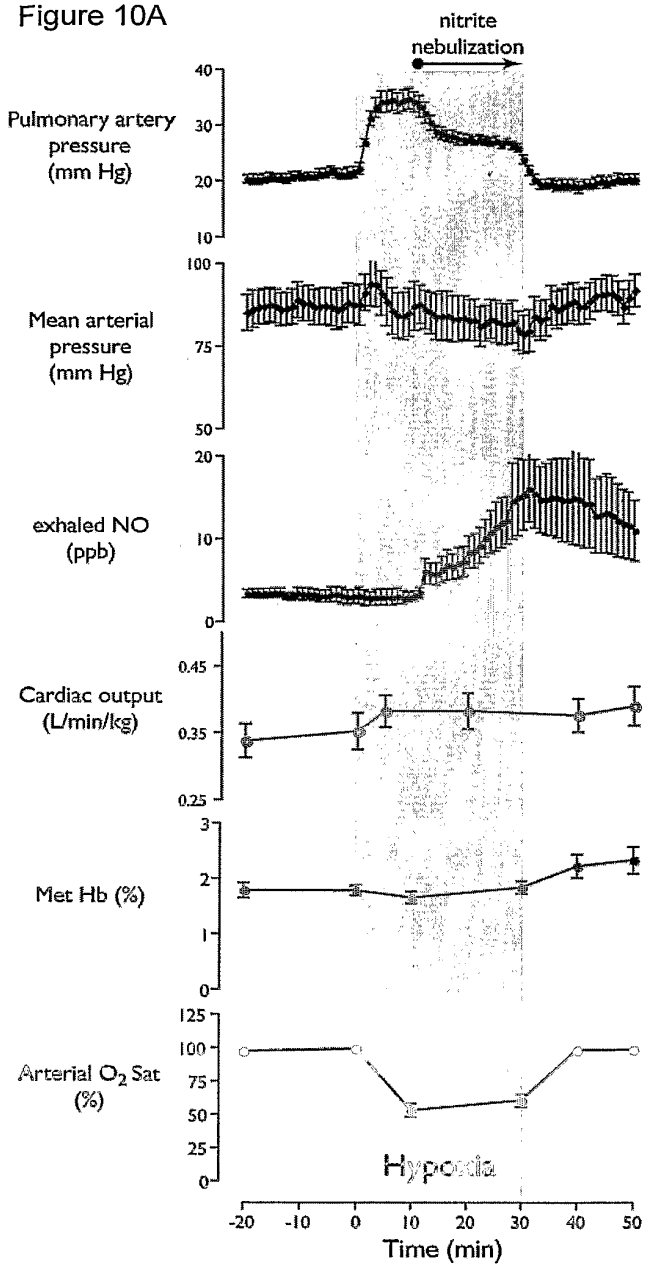


Figure 10B

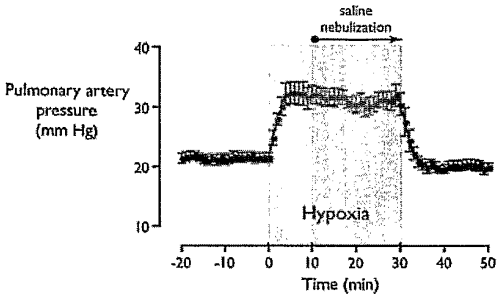


Figure 10C

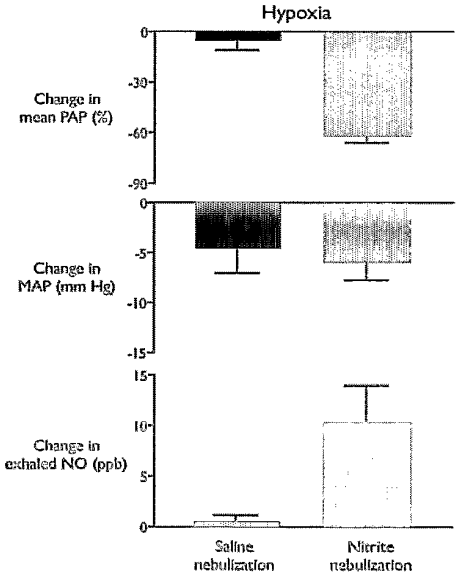


Figure 11

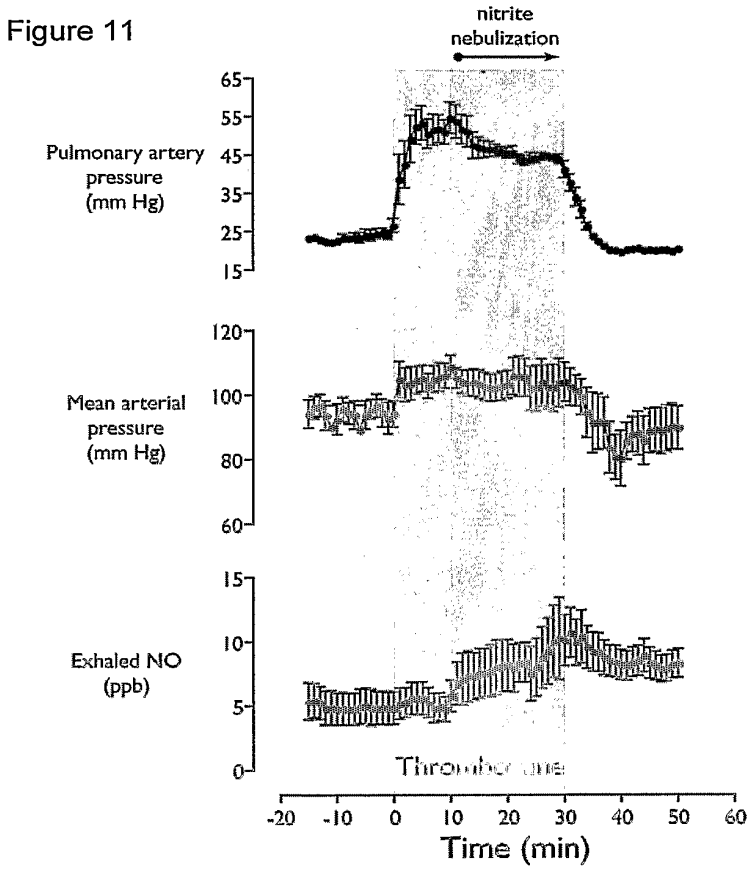

nitrite
nebulization
Pulmonary artery
pressure
(mm Hg)
65
55
45
35
25
15
Mean arterial
pressure
(mm Hg)
120
100
80
60
Exhaled NO
(ppb)
15
10
5
0
-20
-10
0
10
20
30
40
50
60
Time (min)

Figure 12A

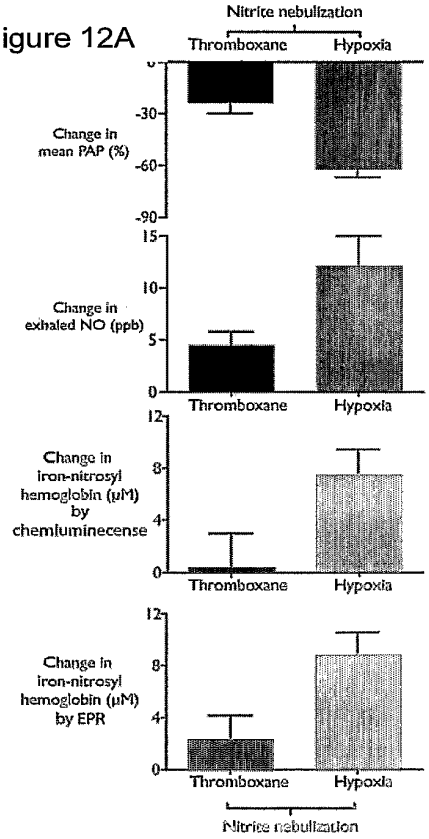


Figure 12B

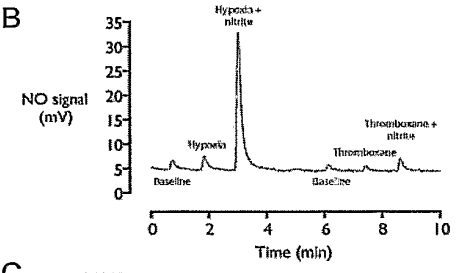


Figure 12C

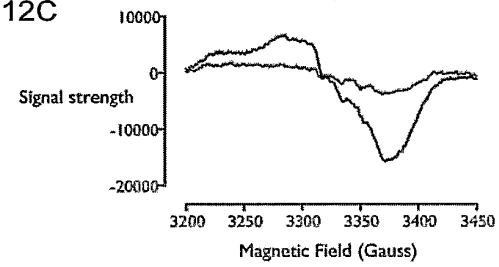
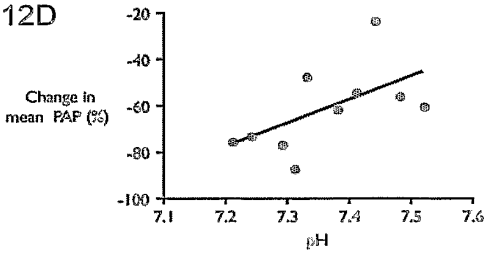


Figure 12D



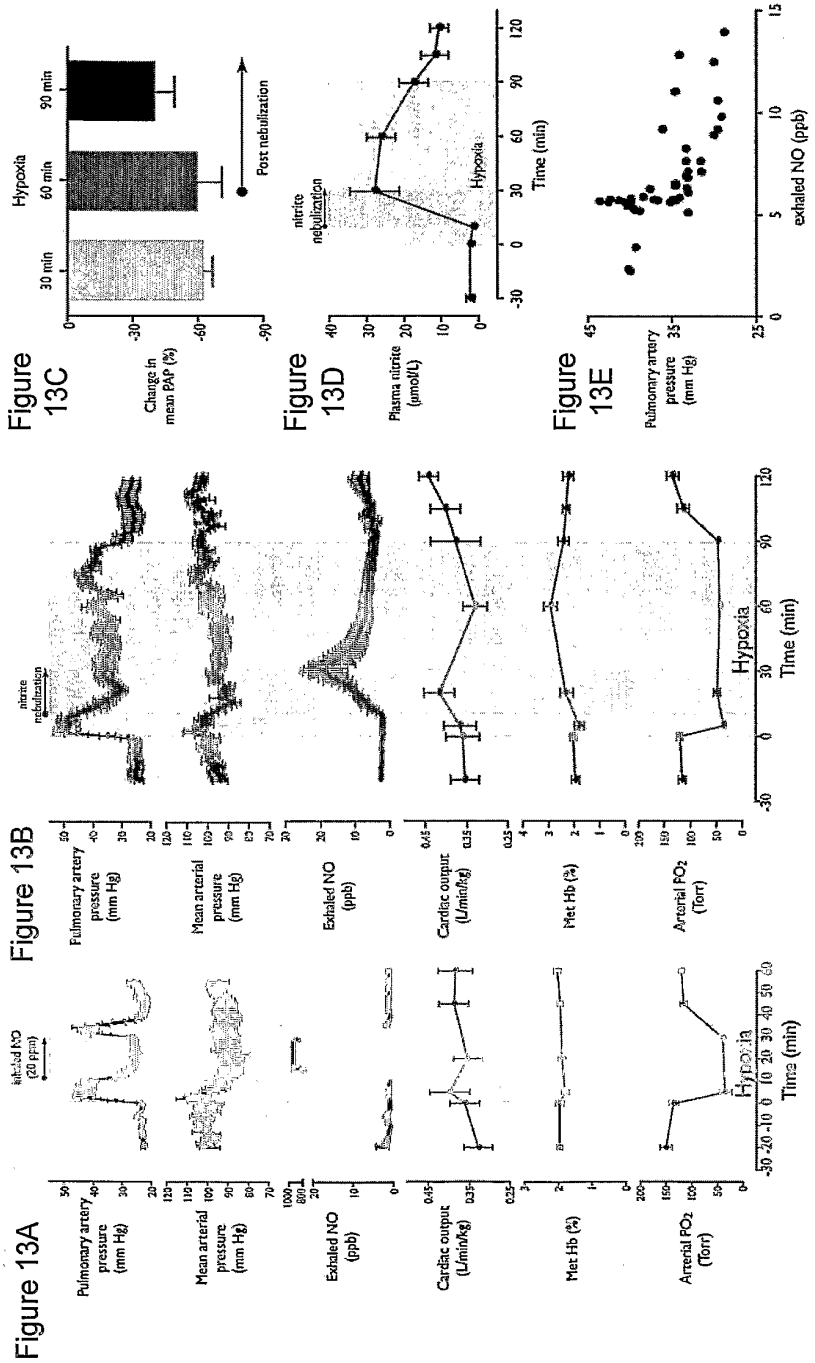
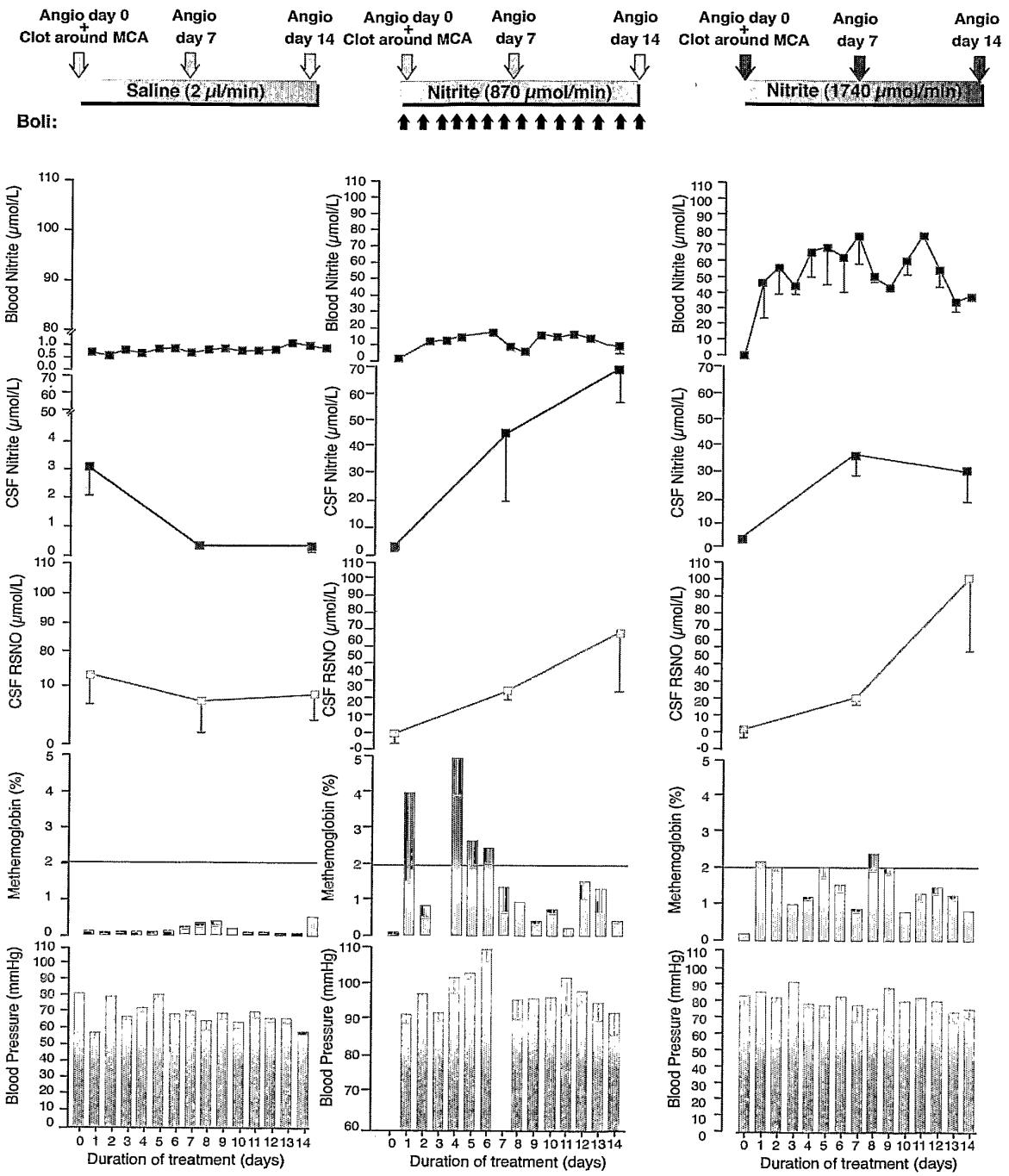


Figure 13A
Inhaled NO (20 ppm)
Pulmonary artery pressure (mm Hg)
Mean arterial pressure (mm Hg)
Exhaled NO (ppb)
Cardiac output (L/min/kg)
Met Hb (%)
Arterial PO2 (Torr)
Hypoxia
Time (min)
Figure 13B
nitrite nebulization
Pulmonary artery pressure (mm Hg)
Mean arterial pressure (mm Hg)
Exhaled NO (ppb)
Cardiac output (L/min/kg)
Met Hb (%)
Arterial PO2 (Torr)
Hypoxia
Time (min)
Figure 13C
Hypoxia
30 min
60 min
90 min
Post nebulization
Change in mean PAP (%)
Figure 13D
nitrite nebulization
Hypoxia
Time (min)
Plasma nitrite (μmol/L)
Figure 13E
Pulmonary artery pressure (mm Hg)
exhaled NO (ppb)

Figure 14


Angio day 0
+
Clot around MCA
Angio
day 7
Angio
day 14
Saline (2 µl/min)
Nitrite (870 µmol/min)
Nitrite (1740 µmol/min)
Boli:
Blood Nitrite (µmol/L)
CSF Nitrite (µmol/L)
CSF RSNO (µmol/L)
Methemoglobin (%)
Blood Pressure (mmHg)
Duration of treatment (days)

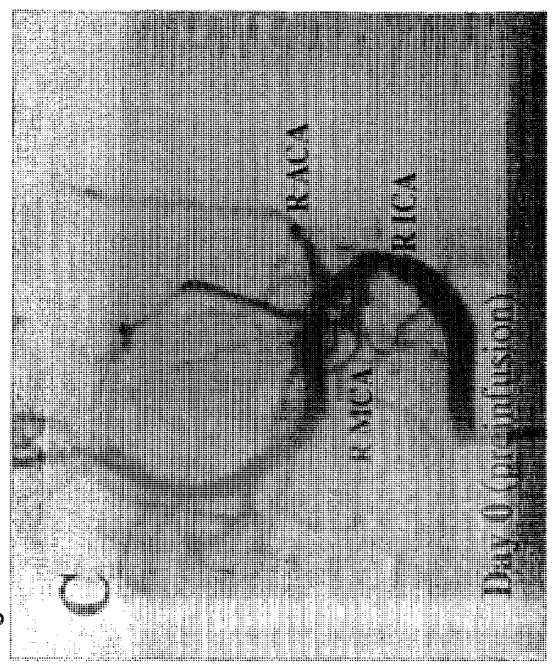


Figure 15C

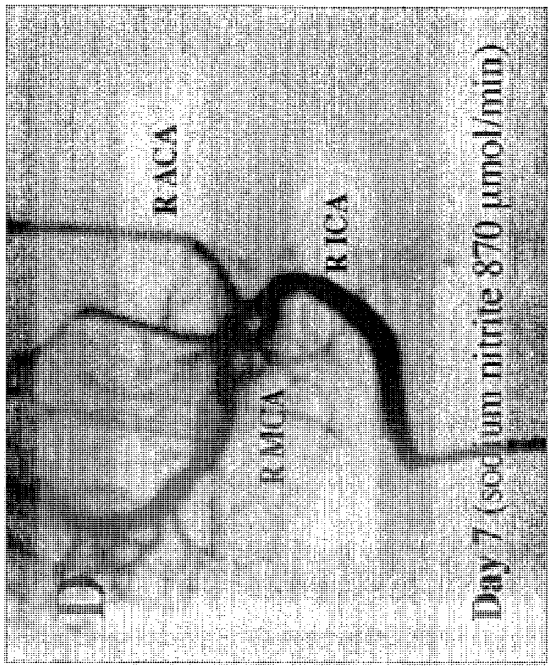


Figure 15D

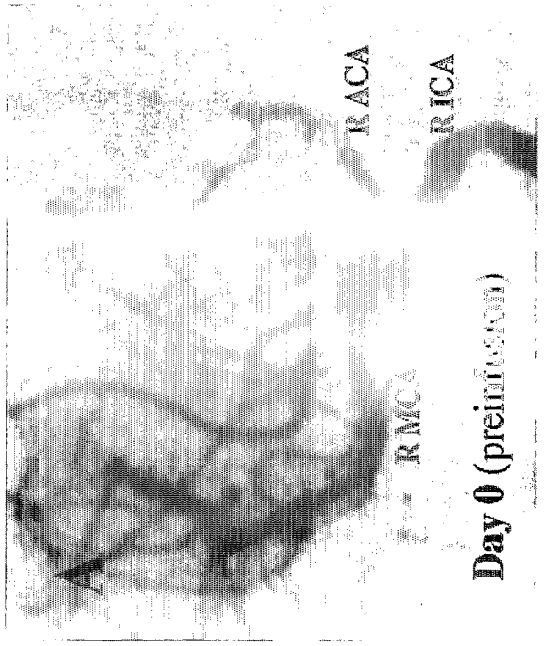


Figure 15A

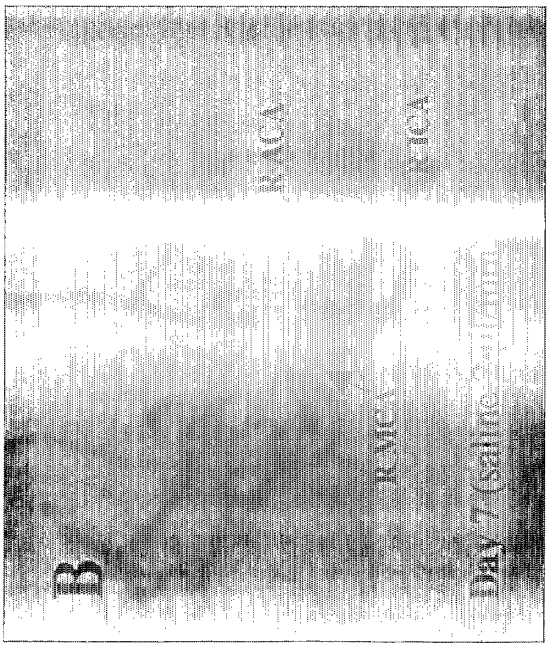


Figure 15B

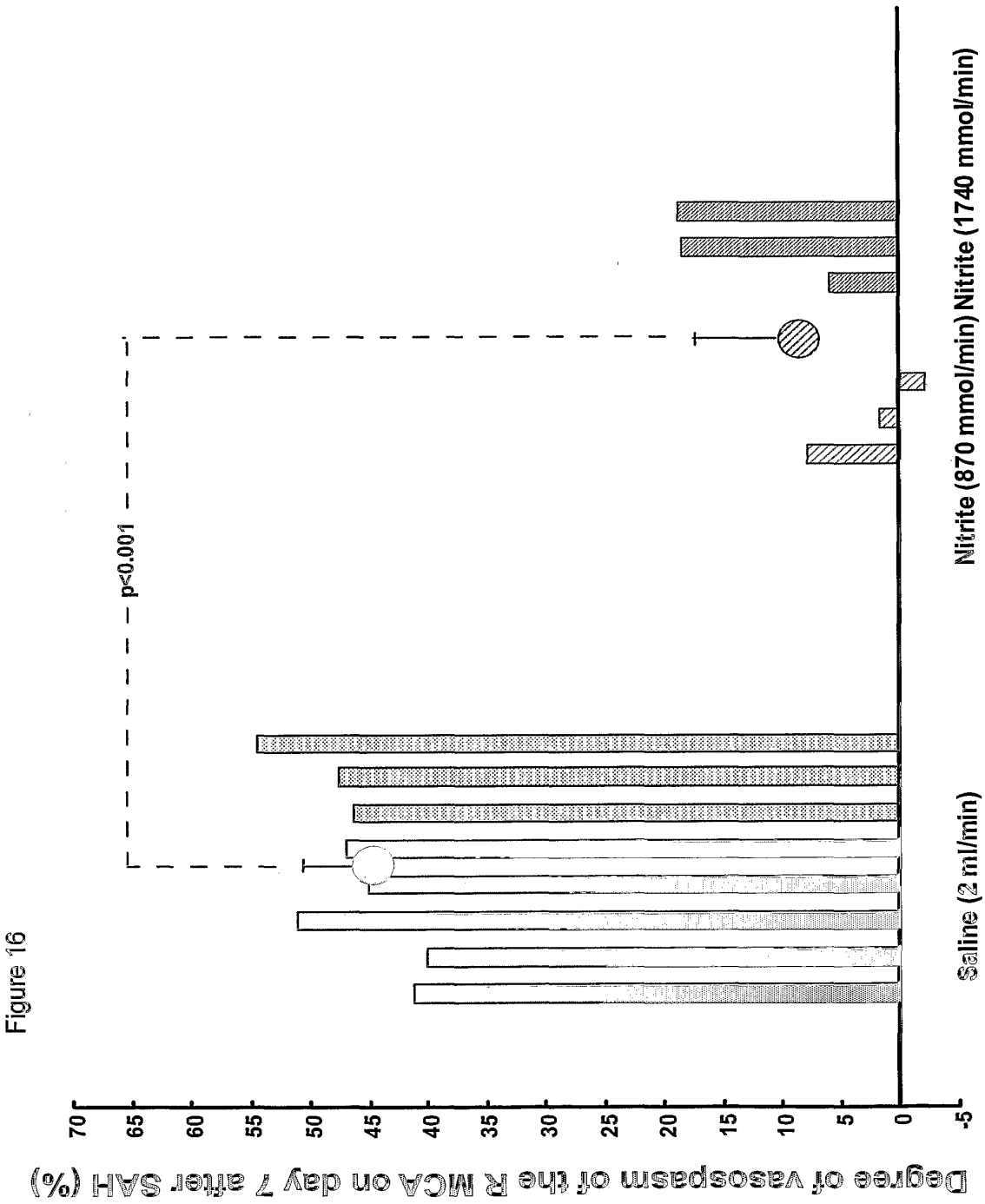

Degree of vasospasm of the R MCA on day 7 after SAH (%)
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
-5
Saline (2 ml/min)
Nitrite (870 mmol/min)
Nitrite (1740 mmol/min)
p<0.001

Figure 16

WO2006127907

Publication Title:

LOCALIZED DELIVERY OF CARDIAC INOTROPIC AGENTS

Abstract:

Abstract of WO 2006127907

(A2) Translate this text The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, such as the ventricles, for example in a subject undergoing cardiothoracic surgery, with the aim of supporting the myocardial contractile function of the heart.

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(71) Applicant *(for all designated States except US)*: **MASSACHUSETTS INSTITUTE OF TECHNOLOGY** [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **EDELMAN, Elazer** [US/US]; 30 Warren Street, Brookline, MA 02445 (US). **LOVICH, Mark** [US/US]; 30 Griggs Road, Brookline, MA 02446 (US).

(74) Agents: **EISENSTEIN, Ronald, I.** et al.; NIXON PEABODY LLP, 100 Summer Street, Boston, MA 02110 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
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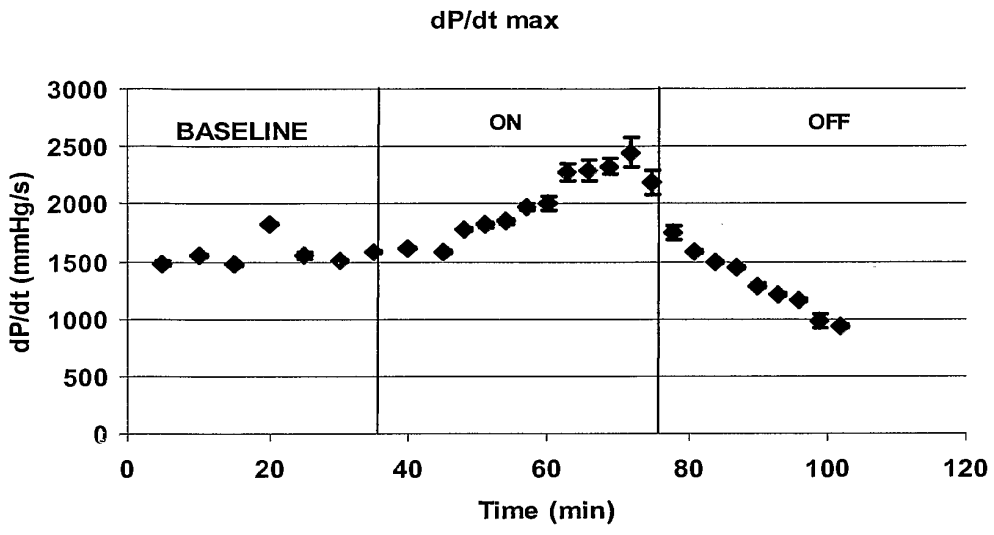
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: LOCALIZED DELIVERY OF CARDIAC INOTROPIC AGENTS



(57) Abstract: The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, such as the ventricles, for example in a subject undergoing cardiothoracic surgery, with the aim of supporting the myocardial contractile function of the heart.

WO 2006/127907 A2

LOCALIZED DELIVERY OF CARDIAC INOTROPIC AGENTS

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Serial No. 60/684,594 filed May 25, 2005, the contents of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

[002] The present invention is directed to methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart such as the ventricles, in a subject in need of such contractile support.

BACKGROUND OF THE INVENTION

[003] Performance of cardiac surgery is a delicate and invasive procedure. The majority of epicardial bypass graft surgeries, and all open heart procedures, require temporary arrest of the heart to allow the surgeon to accomplish the required task without interference from heart movement. An extracorporeal machine, known as a cardiopulmonary bypass (CPB) circuit, assumes the heart and lungs' role of supplying oxygenated blood to the rest of the body while the heart is arrested. Once the surgery is completed, the heart must be re-started, and the patient weaned from the CPB.

[004] While the use of CPB makes cardiac surgery feasible, it is also associated with significant risks and difficulties. The use of a CPB machine usually requires an aortic cross-clamp to separate the heart from the rest of the circulation. Because the coronary arteries arise very close to the heart, the cross clamp must be applied distal to their ostia and therefore they receive no blood flow for prolonged periods, and thus the heart becomes ischemic. Despite numerous myocardial protection strategies, such as hypothermia and chemical cardioplegia to decrease oxygen consumption by arresting the heart, many patients' heart function is

significantly impaired by both chemical arrest and the CPB circuit itself. Chemical cadioplegia, altered coronary perfusion, embolic events and direct manual manipulation of the heart during the procedure all contribute to depression of myocardial function after it is restarted. Furthermore, the degree of post CPB dysfunction may depend on the duration of the CPB time. Patients emerge from chemical cardiac arrest with a spectrum of left ventricular dysfunction, from transient mild impairment to outright ventricular failure and inability to be separated from the CPB. Patients with preexisting ventricular dysfunction are at the greatest risk for further myocardial impairment during CPB.

[005] Moreover, because of improvements in surgical technique and intraoperative myocardial protection, as well as the increasing availability of sophisticated valvular, direct myocardial resections, repairs of septal defects, and coronary bypass procedures, more cardiac operations are being performed on patients with more advanced stages of disease and decreased ventricular function. Indeed, the number of operative risk factors, including advanced age, female gender, severity of angina, triple vessel disease, and left ventricular dysfunction, has increased among patients currently undergoing coronary artery bypass surgery [Davis PK, et al., Ann Thorac Surg 1989; 47:493-98].

[006] In addition, there are important, potentially damaging effects of CPB itself on the cardiovascular system, including increased capillary permeability with attendant transcapillary plasma loss, renal dysfunction, peripheral or central vasoconstriction, coagulopathy, platelet destruction and dysfunction, and destruction of red blood cells [Kalter RD, et al., .J Thorac Cardiovasc Surg 1979; 77:428-35; Kirklin JK, et al., J. Thorac Cardiovasc Surg 1983; 86:845-57.]. Patients with preexisting cardiomyopathies are at even greater risk for postoperative contractile dysfunction. These effects are often transient, but their timing and intensity can make it difficult to impossible in many instances to separate the patient from the CPB circuit.

[007] Weaning a patient off cardiopulmonary bypass (CPB) is a critical step of cardiac surgical procedures. Restarting the heart and returning it and the lungs to the circulation after CPB carries the potential to severely stress an already compromised heart. In the best of circumstances, weaning off CPB can be a relatively straightforward process that requires reestablishing ventilation to the lungs and slowly lowering the circulatory support from the CPB pump. In a significant number of cases however, weaning is especially difficult, and in a few situations simply impossible.

[008] Current available options to support patients who fail to wean from CPB, in order of increasing invasiveness and associated morbidity, include intravenous infusion of inotropes that enhance myocardial contractility, insertion of an intra-aortic balloon pump to augment coronary perfusion and diminish the workload on the heart, and placement of a ventricular assist device. However, each of these treatments is accompanied by significant morbidity and technical limitations, and potential toxicity. Examples of limitations associated with such treatments include proarrhythmic and systemic effects from systemic infusion of inotropes, damage from large-bore indwelling vascular access, need for patient immobility and sedation, as well as risks associated with the placement of a large mass of foreign materials with externalized connections. The pumps and devices have high rates of infection and thromboembolic complications, and require patient immobility, sedation, sometimes prolonged postoperative ventilation, and the most extreme of intensive care nursing support. Weaning of small children after prolonged, difficult and complex operations can represent a further challenge to the surgical team as assist devices may not be readily available in appropriate sizes.

[009] One of the significant challenges of supporting patients as they transition from CPB to the intensive care unit is the variability between patients regarding the timing and degree of support each patient requires. Many patients only need short-term inotropic support to help them transition from CPB to the intensive care unit, while the support required by other patients is much more extensive and

potentially associated with greater risks. Thus, it would be desirable to have less intrusive means that could be used to support these patients as they transition off CPB.

[0010] Inotropic agents are one approach used to enhance a high-risk patient's ability to wean from CPB. Pharmacologic inotropic agents enhance myocardial contractility, and fall into two broad categories: sympathomimetics such as epinephrine (adrenaline), norepinephrine (noradrenaline), dobutamine, isopreterenol, salbutamol, salmeterol, terbutaline, isoproterenol, phenylephrine, ephedrine, clonidine and dopamine, and phosphodiesterase inhibitors such as milrinone and amrinone. Each of these compounds, while increasing the inotropic state of the heart, has limitations that restrict the doses that can be given intravenously and often necessitate infusion of additional agents to counteract side effects. For example, dopamine dosing is limited by the increase in the rate and irritability of electrical excitation of the heart that accompanies the desired inotropic effect. Alternatively, phosphodiesterase inhibitors increase intracellular cyclic AMP, an intracellular signaling molecule that increases inotropy, but unfortunately dilates arterioles and causes systemic vasodilation and hypotension. As a result, vasoconstricting sympathomimetic agents often need to be co-administered and these again can lead to proarrythmogenic states and undesirable tachycardia.

[0011] One important consideration of the use of inotropic agents is that they are administered systemically and thus treat all vascular beds. Systemic side effects of sympathomimetics include potential renal and cerebral vasoconstriction, and pulmonary artery hypertension, which in turn can induce right heart failure. Other undesired effects are excess tachycardia and electrical irritability.

[0012] Accordingly, there is a need for improved methods to support patients as they transition off CPB, by improving contractile function of the heart without extraventricular effects, such as tachycardia, vasoconstriction or systemic hypotension.

SUMMARY OF THE INVENTION

[0013] The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, such as the ventricles, in a subject in need thereof.

[0014] Support of the weakened heart such as occurs while a patient is coupled to a CPB circuit, and while the patient transitions off CPB, is critical to recovery from cardiac surgery. We have discovered methods to take advantage of existing polymeric controlled release strategies to deliver inotropic agents directly or indirectly to the heart, preferably directly, including to specific regions of the heart. By locally delivering the inotropic agent directly to the heart, the systemic exposure of the inotropic agents is limited, avoiding the alterations in vascular tone, and heart rate and electrical excitability associated with systemic administration of these agents.

[0015] The methods of the present invention can be used to treat any patient in need of transient contractile support to the heart, where such support can be provided by the local delivery of inotropic agents either directly or indirectly to the heart, including specific regions of the heart, such as the ventricles. One would apply the agent through the cardiac blood stream, or preferably directly in the heart. The agent can be applied through the coronary artery or vein and onto the heart surface. The agent can also be applied through the ventricular or atrial walls and onto the heart surface. The agent can also be applied through direct and extensive surgical field exposure, minimally invasive exposure via a pericardial window or heart port, or percutaneous or endovascular catheters.

[0016] In one embodiment, the patient is in need of localized delivery of an inotropic agent to provide contractile support as a result of a surgical intervention. Surgical interventions include but are not limited to cardiac surgery, thoracic surgery,

and general surgery. In another embodiment, the patient is in need of transient localized delivery of an inotropic agent to provide contractile support as a result of trauma, shock, or heart failure.

[0017] In another embodiment, the patient is in need of transient inotropic support following an intervention less invasive than a major surgical intervention, referred to herein as a minimally invasive intervention. Such minimally invasive interventions include but are not limited to a percutaneous intervention or a catheter based intervention. In such embodiments, the inotropic agent can be delivered either from inside the heart chamber or from outside the heart.

[0018] One preferred embodiment provides transient localized delivery of inotropic agents to support the heart of a patient undergoing surgery. In one embodiment, the patient requires support from a cardiopulmonary bypass (CPB) circuit. In another embodiment, the patient does not require support from a CPB circuit. In one particularly preferred embodiment, the patient is a cardiac patient.

[0019] The present invention provides the local delivery of any inotropic agent, including but not limited to sympathomimetics and phosphodiesterase inhibitors. Preferred sympathomimetics include epinephrine, norepinephrine, isoproterenol, dobutamine and dopamine, and analogues and derivatives thereof. Preferred phosphdiesterase inhibitors include milrinone and amrinone, and analogues and derivatives thereof.

[0020] Any delivery vehicle which can be loaded with an inotropic agent and directly applied to the heart can be used in the present invention. Delivery vehicles include drug-impregnated, coated or relasing sheets, patches, matrix, hydrogel, foam, gel, cream, spray, microshpere, microcapsule, composite and ointment. Certain preferred delivery vehicles are polymeric controlled release vehicles.

[0021] The delivery vehicle is loaded with the inotropic agent and locally applied to the heart using any route for application which allows its local application

to the heart. In one embodiment, the delivery vehicle may be applied directly to the exposed heart during a surgical intervention, for example before the pericardium or sternum is closed. In another embodiment, the delivery vehicle may be applied through a less direct route, including but not limited to a percutaneous application or an endovascular injection.

[0022] Certain embodiments of the invention provide further localization of the delivery of the inotropic agent. In one embodiment, the delivery vehicle is placed away from the sinoatrial node or the right atrium. A preferred placement of the delivery vehicle is on the left ventricular free wall or apex of the ventricle.

[0023] One particularly preferred embodiment provides local delivery of dopamine to the ventricle without targeting the sinus node in the right atrium, limiting the excessive tachycardia observed in high dose intravenous infusion of this agent.

[0024] Another embodiment of the invention provides the use of a non-permeable barrier on the surfaces of the heart not treated with the delivery vehicle, to achieve additional localization. In another embodiment of the invention, non-permeable barriers can be used to direct drug toward the myocardium and prevent the loss of drug to ventricular blood flow or pericardial fluid.

[0025] Preferably, the delivery methods of the present invention are administered to the subject for a short time, i.e. just long enough to support the heart until it recovers from its weakened condition. Administration of the inotropic agent may last for a few hours to days, for example up to 14 days. The delivery methods of the present invention can be used to treat the heart prior to surgery, during surgery, after surgery, and any combination thereof.

DESCRIPTION OF THE FIGURES

[0026] Figure 1: Figure 1 shows contractility of the heart (max dp/dt (mmHg/s)) over time in rats administered dobutamine, a non selective beta agonist inotropic agent, which was delivered directly to the left ventricular wall. Contractility

was significantly increased shortly after dobutamine was applied to the surface of the heart.

[0027] Figure 2: Figure 2 shows left ventricular systolic blood pressure over time in rats administered Dobutamine, a nonselective beta agonist inotropic agent, which was delivered directly to the left ventricular wall. Local pericardial delivery of dobutamine increased systemic blood pressure. It is known that intravenous infusion of inotropic agents reduce systemic blood pressure.

[0028] Figure 3: Figure 3 shows heart rate over time in rats administered Dobutamine, a non selective beta agonist inotropic agent, which was delivered directly to the left ventricular wall.

DETAILED DESCRIPTION OF THE INVENTION

[0029] The present invention provides novel methods for the localized delivery of inotropic agents to the heart, including specific regions of the heart, in a subject in need of transient contractile support. One embodiment provides localized delivery of inotropic agents to support the heart during or following cardiac surgery, including as a subject transitions off of a cardiopulmonary bypass (CPB) circuit.

[0030] The present invention provides advantages over known methods to support the weakened heart, such as while a cardiac surgery patient is coupled to a CPB circuit, and as a patient transitions off CPB. To avoid the adverse side effects associated with systemic delivery of positive inotropic agents, we have discovered methods to take advantage of existing polymeric controlled release strategies to locally deliver inotropic agents directly to the heart. By locally delivering the inotropic agent directly to the heart, the systemic exposure of the inotropic agents is limited, avoiding the peripheral arterial dilation and systemic hypotension associated with systemic administration of some of these agents, and the tachycardia and vasoconstriction associated with others. In addition, because the methods of the

invention deliver the positive inotropic agent directly to localized heart surface, lower amounts, but potentially high local concentrations, can be delivered.

[0031] The inventors of the present invention have surprisingly shown that inotropic agents, when applied directly to the heart rather than systemically, increase contractility of the heart and minimize systemic side effects such as the reduction in systemic blood pressure that is seen when certain inotropic agents, such as dobutamine, isoproterenol, milrinone, or amrinone are administered systemically. Thus, the inventors have shown that local delivery of inotropic agents mimimizes systemic side effects while improving contractile function of the heart.

[0032] In one embodiment, a method of locally delivering a cardiac inotropic agent to the heart of a subject is encompassed. This method comprises locally administering to a subject in need thereof a therapeutically effective amount of at least one inotropic agent.

[0033] In one embodiment, the inotropic compound is an agent that interacts with the sympathetic nervous system and modulates calcium entry, G-proteins, ATP, or GTP, wherein the inotropic agent is selected from the group consisting of sympathomimetic compounds, phosphodiesterase inhibitors, BNP, ANP, and digitalis glycosides, and derivatives and analogues thereof.

[0034] The inotropic agent may be a sympathomimetic compound selected from the group consisting of epinephrine, norepinephrine, dobutamine, isoproterenol, salbutamol, salmeterol, terbutaline, phenylephrine, ephedrine, clonidine and dopamine, and derivatives and analogues thereof.

[0035] Alternatively, the inotropic agent may be a phosphodiesterase inhibitor selected from the group consisting of milrinone and amrinone, and derivatives and analogues thereof.

[0036] The subject to be treated may be a surgical patient. Non-limiting examples of surgical patients are a cardiac surgery patient, a thoracic surgery patient, and a general surgery patient.

[0037] In one embodiment, the cardiac surgery patient is selected from the group consisting of a cardiac surgery patient requiring support from a cardiopulmonary bypass circuit and a cardiac patient not requiring support from a cardiopulmonary bypass circuit.

[0038] In another embodiment, the subject has a condition selected from the group consisting of trauma, shock, and congestive heart failure.

[0039] In one embodiment, the inotropic agent is locally delivered to the heart by administering the inotropic agent directly to the heart via an open surgical wound. Alternatively, the local delivery comprises administering said inotropic agent directly to the heart percutaneously.

[0040] A method of reducing postoperative complications of cardiopulmonary bypass (CPB) surgery in a subject is also encompassed in the present invention. This method comprises locally administering to a subject in need thereof an effective amount of an inotropic agent in conjunction with CPB surgery of said subject. The inotropic agent may be a sympathomimetic compound or a phosphodiesterase inhibitor.

[0041] The inotropic agent may be administered to said subject during a time period consisting of 1) prior to said CPB surgery; 2) during said CPB surgery; 3) subsequent to said CPB surgery; and 4) combinations thereof.

[0042] As used herein, a "therapeutically effective amount" of the inotropic agent is an amount that is sufficient to effect myocardial contractility.

[0043] The inotropic agent may be delivered locally to the heart by its inclusion in a delivery vehicle.

Subjects for Administration

[0044] The methods of the present invention can be used to treat any patient in need of transient contractile support to the heart, where such support can be provided by the local delivery of inotropic agents directly to the heart, including specific regions of the heart, such as the ventricles.

[0045] In one embodiment, the patient is in need of localized delivery of an inotropic agent to provide transient contractile support as a result of a surgical intervention. Surgical interventions include major surgeries, including but not limited to cardiac surgery, thoracic surgery, and general surgery. In another embodiment, the patient is in need of localized delivery of an inotropic agent to provide contractile support as a result of trauma, shock, or heart failure.

[0046] In another embodiment, the patient is in need of inotropic support following an intervention less invasive than a major surgical intervention, referred to herein as a minimally invasive intervention. Such minimally invasive interventions include but are not limited to a percutaneous intervention or a catheter based intervention. In such embodiments, the inotropic agent can be delivered either from inside the heart chamber or from outside the heart, as described in detail below.

[0047] One preferred embodiment provides localized delivery of inotropic agents to support a patient undergoing surgery. In one embodiment, the surgical procedure requires the use of a cardiopulmonary bypass (CPB) circuit. In another embodiment, the procedure does not require the use of a CPB circuit. In one particularly preferred embodiment, the patient is a cardiac patient.

[0048] In order to perform many surgical procedures it is necessary to interrupt coronary blood flow. Without cardioprotective strategies such as cooling and chemical arrest, the heart would soon die. Unfortunately, no cardioprotective strategy has been shown to be optimal and some degree of post CPB contractile dysfunction is inevitable. This is not only a problem in the adult patient undergoing

coronary artery bypass surgery (CABG) or other surgical procedures, it is also a significant clinical problem during surgical heart procedures to correct congenital heart defects in neonates.

[0049] Thus, local administration of the agent can begin at any time once surgery begins until twenty-four hours after surgery has ended. More typically, within 12 hours of surgery ending. Any range within these ranges can be used, such as 1, 2, 3, 4, or more hours after surgery has ended.

[0050] In certain embodiments, administration of the agent can begin before surgery, for example using a percutaneous approach for delivery of the agent.

[0051] Accordingly, the methods of the present invention can be used to treat any subject while coupled to a CPB circuit, i.e. during cardiac surgery, and/or following cardiac surgery, during their transition off of the CPB circuit. Cardiac surgery includes any surgical procedure on the heart and usually involves interruption of coronary blood flow. It can also be used to assist the heart function during and after any thoracic surgical procedure where the heart is already exposed to the surgeon.

[0052] Before turning the CPB circuit, also known as the pump, off, all clinical determinants of cardiac performance are evaluated and adjusted, in order to optimize cardiac output. All metabolic, thermal, electrolyte, acid/base, and hematologic abnormalities are corrected. Blood volume is adjusted according to central venous, left atrial or pulmonary artery pressures. Peripheral resistance is estimated and vasodilators or constrictors are instituted as required. After the drug's effectiveness is assessed, pump flow is decreased in small increments while venous return to the heart is proportionately adjusted to maintain a constant filling pressure by constricting the venous drainage to the CPB circuit.

[0053] The assessment of cardiac function by transesophageal echocardiography and hemodynamic data immediately before terminating CPB allows

patients to be classified into 3 groups by decreasing risk, referred to herein as groups A, B, and C [Souza et al., Indian Journal of Extracorporeal Technology 6:2, 1998]. The methods of the present invention can be used to treat any patient in group A, B, or C, including children in need of inotropic support during cardiac surgery or during weaning from CPB.

[0054] The highest risk patients, classified herein as "Group A" patients, have severe cardiac dysfunction that makes it difficult to be removed from CPB, despite physiologic and pharmacological support. For these patients CPB is prolonged. Group A patients are by definition the hardest cases to manage. A few of these patients by the end of rewarming of the blood will have minimal or no cardiac activity, which precludes any trial of disconnection from pump. The remaining patients may be given a short trial off pump after optimization of preload, afterload and contractility by a combination of inotropes and vasoactive agents. Some of these patients will tolerate CPB removal, under maximal physiological and pharmacological support, and a few in the group may be further improved by an intra-aortic balloon pump. The patients with minimal cardiac activity and those in whom the trial off pump was unsuccessful are temporarily maintained on cardiac support with the heart-lung machine. A few hours on pump support may be a sufficient rest period to allow recovery of cardiac function and removal of CPB support in a small number of cases. For the others, a decision has to be made as to either advance to a mechanical device for prolonged support or terminate the efforts to recover cardiac action.

[0055] Children in Group A supported by full veno-arterial extracorporeal membrane oxygenation (ECMO) post cardiotomy have a poor long term survival rate [Langley et al., Eur J Cardiothorac Surg 13, 520-5, 1998] when compared with children managed with centrifugal ventricular assist devices [Thuys et al., Eur J Cardiothorac Surg 13, 130-4, 1998]. The methods of the present invention may be utilized in the treatment of children in Group A.

[0056] In certain cases, a few hours of circulatory assistance and intensive inotropic and vasodilator drug therapy may turn some Group A patients into group B. The remaining patients are candidates to a form of total circulatory mechanical support (if available) or they will not likely survive disconnection from pump [Harris C. et al., Tecnol. Extracorp. Rev. Latinoamer. 3, 13-19, 1996; El-Banayosy A., et al., Perfusion, 11, 93-102, 1996; Núñez HI., Tecnol. Extracorp. Rev. Latinoamer. 2, 33-41, 1995].

[0057] Group B patients have a mild to moderate degree of cardiac dysfunction, and require greater support and a more elaborate protocol for CPB termination than patients in Group C. Final preparations are made on partial bypass. In addition to the delivery of inotropic agents using the present invention, these patients may also be supported by physiological means such as volume resuscitation or additional pharmacological means, namely vasodilators. Some patients in this group can benefit from intra-aortic balloon pumping. Patients in this group will benefit from the methods of the present invention.

[0058] Some Group B patients may have to return to pump for better adjustment of drugs, or to have an intra-aortic balloon inserted if a marginal cardiac output is present, as demonstrated by atrial and arterial pressures, arterial and venous blood gases and pH, and spontaneous diuresis.

[0059] Group B patients include children with preoperative intracardiac shunts leading to high pulmonary blood flow, children after a heart transplant, and some adults with long standing congestive heart failure, who may present with pulmonary hypertension that precludes successful weaning. In certain instances, inhalation of nitric oxide (NO) can improve pulmonary hypertension and cardiac output and support discontinuance of CPB. Additional Group B patients include patients who received inadequate myocardial protection for any reason, including inadequate re-dosing of cardioplegia, inadequate perfusion of myocardia with

cardioplegia, patients with severe ventricular hypertrophy or aortic insufficiency, surgical errors, and prolonged CPB time.

[0060] An occasional patient in group B will not tolerate CPB termination even after a few trials. These few exceptions turn into group A patients.

[0061] For lower risk "Group C" patients, inotropic support of the present invention can be provided at a lower level, and may be discontinued as the patient arrives at the intensive care area or a few hours thereafter. The methods of the present invention can be used as needed to treat Group C patients, who are anticipated to smoothly disconnect from perfusion. For these patients, after reestablishing ventilation to the lungs, pump flow can be gradually reduced while venous return to the oxygenator is decreased until bypass is minimal. Arterial pump is stopped and venous line is clamped. Final adjustment of cardiac performance is made off pump, by slowly administering residual volume from the oxygenator until ideal preload is attained. These patients maintain an adequate cardiac output, as can be confirmed by normal atrial and arterial pressures, arterial and venous blood gases and pH and adequate spontaneous diuresis.

[0062] In one particularly preferred embodiment of the invention, the methods can be used to treat any subject undergoing non cardiac thoracic surgery where the heart is exposed, to assist the heart function and/or to treat contractile dysfunction.

[0063] In some embodiments, the inotropic agent of the present invention can be co-administered with prostaglandin E1, which can act as a powerful adjunct to wean difficult transplanted children with right ventricular failure.

[0064] In some embodiments, the inotropic agent of the present invention can be co-administered with nitroprusside or other vasodilator drugs.

[0065] In some embodiments, one particularly preferred inotrope is enoximone, to provide pharmacological support during weaning of patients with severe ventricular dysfunction.

[0066] The term "subject" as used herein refers to vertebrates, particularly members of the mammalian species and includes but is not limited to, domestic animals, sports animals, primates, dogs, cats, rodents including mouse and rat, horse and humans; more preferably, the term refers to humans.

Inotropic Agents

[0067] As used herein, "inotropic agents" or "positive inotropic agents" or "inotropes" or "positive inotropes" or "inotropic antibodies" will be used interchangeably and refers to the effect such agents produce, i.e. improves cardiac output by increasing the force of myocardial muscle contraction. "Positive inotropic effect" means that the contractility of the cells is enhanced in a dose-dependent manner. A positive inotropic effect-producing amount of an inotropic agent of the invention can be administered to a subject.

[0068] Positive inotropic agents of the present invention include any agents which provide the heart with contractile support. The agent can be an inotropic agent such as a sympathomimetic or a phosphodiesterase inhibitor, as long as one obtains the desired contractile effect on the heart. Inotropic compounds include agents that interact with the sympathetic nervous system and modulate calcium entry, G-proteins, ATP and GTP. Inotropic compounds include sympathomimetic compounds, phosphodiesterase inhibitors, BNP, ANP, and digitalis glycosides. Preferably, the agent is a sympathomimetic or a phosphodiesterase inhibitor. Preferred sympathomimetics include but are not limited to epinephrine, norepinephrine, dopamine, dobutamine, dopexamine, terbutaline, and isoproterenol, and analogues and derivatives thereof. Preferred phosphdiesterase inhibitors include but are not limited to milrinone, amrinone, enoximone, and pimobendan, and analogues and derivatives thereof.

[0069] Preferably, the positive inotropic agent is administered in the form of a pharmaceutical composition. A pharmaceutical composition comprising an effective amount of the positive inotropic agent as an active ingredient can be prepared by standard procedures well known in the art, with pharmaceutically acceptable non-toxic solvents and/or sterile carriers, if necessary. For example, the inotropic agent can be embedded in a controlled-release polymer. In other embodiments the positive inotropic agent is administered without a pharmaceutical carrier.

[0070] The dose of the positive inotropic agent is a therapeutically effective dose. In particular embodiments, the positive inotropic agent can be administered at a dose which produces in the subject an effect equivalent to the systemic intravenous administration of between 2 and 20 mcg/kg/min. However, in other embodiments, higher and lower dosages can be administered to subjects. For example, a dose which produces in the subject an effect equivalent to the systemic intravenous administration of 0.5 mcg/kg/min, or 40 mcg/kg/min. Optimizing therapy to be effective across a broad population can be performed with a careful understanding of various factors to determine the appropriate therapeutic dose. Typically, the dose can be much lower than the dose administered by intravenous infusion, because the agent is being locally delivered to the heart, rather than systemic administration.

Localization of the Delivery Vehicle on the Heart

[0071] Routes for direct application of the delivery vehicle to the heart include any routes which allow the delivery vehicle to be applied locally to the heart. For example, the delivery vehicle may be applied from the blood stream, by being placed directly in the heart through the coronary arteries or veins onto the heart surface; or through the ventricular or atrial walls and onto the heart surface. The delivery vehicle may also be applied through direct application during extensive surgical field exposure, or through direct application during minimally invasive exposure, for example through a pericardial window or heart port. The delivery

vehicle may also be applied through a percutaneous route, or via endovascular catheters.

[0072] In one embodiment, the delivery vehicle is loaded with the inotropic agent and placed over the heart of a surgical patient, before the sternum is closed, allowing direct release of the inotropic agent to the heart.

[0073] Placement of the delivery vehicle can be understood with reference to the different compartments of the heart. The heart is subdivided by a muscular septum into two lateral halves, which are named respectively right and left. A transverse constriction subdivides each half of the heart into two cavities, or chambers. The upper chambers consist of the left and right atria, which collect blood and help fill the lower chambers. The lower chambers consist of the left and right ventricles, which pump blood to the rest of the body. The chambers are defined by the epicardial wall of the heart. The right atrium communicates with the right ventricle by the tricuspid valve. The left atrium communicates with the left ventricle by the mitral valve. The right ventricle empties into the pulmonary artery by way of the pulmonary valve. The left ventricle empties into the aorta by way of the aortic valve.

[0074] The circulation of the heart consists of two components. First is the functional circulation of the heart, i.e., the blood flow through the heart from which blood is pumped to the lungs and the body in general. Second is the coronary circulation, i.e., the blood supply to the structures and muscles of the heart itself. The functional circulation of the heart pumps blood to the body in general, i.e., the systemic circulation, and to the lungs for oxygenation, i.e., the pulmonic and pulmonary circulation. The left side of the heart supplies the systemic circulation. The right side of the heart supplies the lungs with blood for oxygenation. Deoxygenated blood from the systematic circulation is returned to the heart and is supplied to the right atrium by the superior and inferior venae cavae. The heart pumps the deoxygenated blood into the lungs for oxygenation by way of the main pulmonary

artery. The main pulmonary artery separates into the right and left pulmonary arteries, which circulate to the right and left lungs, respectively. Oxygenated blood returns to the heart at the left atrium via four pulmonary veins. The blood then flows to the left ventricle where it is pumped into the aorta, which supplies the body with oxygenated blood.

[0075] The functional circulation supplies blood to the heart by the coronary circulation. The coronary arteries arise from the proximal aorta through the left and right coronary ostia course along the epicardial surface of the heart and send of numerous branches to supply the myocardium. Blood is cleared from the muscle by cardiac veins that flow into the coronary sinus and right atria. The heart wall is surrounded by a pericardial sac, which contains it within interstitial fluid.

[0076] In one embodiment, the delivery vehicle loaded with the inotropic agent is placed over the heart, before the sternum is closed, allowing direct release of the inotropic agent to the heart. In one embodiment, the delivery vehicle is placed away from the sinoatrial node or the right atrium. A preferred placement of the delivery vehicle is on the apex of the ventricle or left ventricular free wall.

[0077] Another embodiment of the invention provides the use of a non-permeable barrier on the surfaces of the heart not treated with the delivery vehicle, to achieve additional localization. In another embodiment, the delivery vehicle itself can be coated with a non-permeable barrier, to further localize release of the agent directly to the underlying heart tissue, while minimizing release into the pericardial fluid.

[0078] One particularly preferred embodiment provides local delivery of dopamine, epinephrine, norepinephrine, isoproterenol, and dobutamine to the ventricle without targeting the sinus node in the right atrium, limiting the excessive tachycardia observed in intravenous infusion.

[0079] In one embodiment, the delivery vehicle contains an inotropic agent that must be activated or released by a second agent. That second agent can be added

systemically to locally activate or release the inotropic agent. In this way, timing and/or release can be controlled at later points.

Treatment Period

[0080] Preferably, the delivery methods of the present invention are administered to the subject just long enough to support the heart until it recovers from its weakened condition. The short term or transient administration of the inotropic agent may last for a period of several minutes to several days. For example, from five minutes to 14 days. Typically, at least two hours to seven days. Preferably five hours to five days. More preferably, 2-24 hours. One can use all ranges between 5 minutes to 14 days, e.g. 12 hours to 12, 11, 10, 9, 8, 7, or fewer days.

[0081] In one embodiment of the invention, the patient is a surgical patient and the delivery methods of the present invention can be used to treat the heart prior to surgery, during surgery, after surgery, and any combination thereof.

Delivery Vehicle

[0082] The delivery vehicle of the present invention is any drug delivery means that can incorporate an inotropic agent, and is suitable for administration directly to the heart for local delivery or release of that agent. Suitability for local delivery to the heart includes the ability of a delivery vehicle to adhere to the underlying tissue. Any delivery vehicle which can be loaded with an inotropic agent and locally applied to the heart can be used in the present invention.

[0083] Examples of delivery vehicles include but are not limited to a patch, a matrix, a hydrogel, a sheet of material, a foam, a gel, a cream, a spray, and an ointment. Certain preferred delivery vehicles are polymeric controlled release vehicles. In one embodiment, the delivery vehicle is a patch, such as a transepicardial patch, that slowly releases the agents directly into the myocardium. In one embodiment, the delivery vehicle is an ointment or cream which may be placed manually on the target area of the heart. In one preferred embodiment, the delivery

vehicle is a hydrogel, which may be polymerized either directly on the heart in vivo or polymerized in vitro to form a patch for administration.

[0084] In one preferred embodiment, the inotropic agent(s) of the invention are incorporated into a biocompatible delivery vehicle referred to as a matrix. The matrix can be in the form of a gel, foam, suspension, microcapsules, solid polymeric support, or fibrous structure. The matrix may also serve in a physically supporting role. There is no specific requirement as to thickness, size or shape. It is preferred that the matrix be sufficiently porous to allow the inotropic agent to diffuse out of the matrix into the surrounding tissue in roughly physiologic quantities.

[0085] Preferably, the matrix is a biodegradable material. Preferably, the hydrogel matrix degrades in a period of time minimizing tissue inflammation, for example in less than seven to ten days. Examples of a biodegradable matrices include but are not limited to synthetic polymers degrading by hydrolysis, for example, polyhydroxy acids like polylactic acid, polyglycolic acid and copolymers thereof, polyorthoesters, polyanhydrides, proteins such as gelatin and collagen, or carbohydrates or polysaccharides such as cellulose and derivatized celluloses, chitosan, alginate, or combinations thereof, so that over the course of several days or weeks after implantation of the matrix material, the matrix gradually disappears.

[0086] The use of biodegradable matrices eliminates the need for surgery to remove undegraded implanted matrix. However, synthetic non-biodegradable matrices may also be used. Useful materials include but are not limited to ethylene vinyl acetate, polyvinyl alcohol, silicone, polyurethane, non-biodegradable polyesters, and polyethyleneoxide-polypropyleneoxide, and tetrafluoroethylene meshes (Teflon®).

[0087] In a preferred embodiment, the matrix is a hydrogel, defined as a matrix wherein typically approximately 900-fold by weight of the matrix is absorbed water. Hydrogels are well known in the art. Hydrogels can be formed by ionic or covalent crosslinking of a variety of water soluble polymers such as

polyphosphazenes, polysaccharides such as alginate, and proteins such as gelatin. For example, one matrix material is purified gelatin-based Gelfoam™ (The Upjohn Co., Kalamazoo, Mich.) surgical sponge.

[0088] To achieve the above properties, the hydrogel is formed primarily of polymerized macromers, the macromers being themselves polymers or copolymers of one or more monomers having reactive groups providing resorbable linkages and polymerizable sites for biodegradability and polymerization. The macromers have sufficient hydrophilic character to form water-absorbent polymerized gel structures, and are at least dispersible in a substantially aqueous solution, and preferably are water-soluble, to maximize tissue adherence. The macromers are preferably made predominantly of synthetic materials. The resulting hydrogels are preferably highly compliant, so as not to impede the process of cardiac contraction. The hydrogels are preferably covalently crosslinked to ensure that they are retained at the site of application until the hydrogels degrade. In certain embodiments, the gel can be crosslinked in situ, for example by photopolymerization.

[0089] Monomers and macromers which are suitable for forming the hydrogels ("referred to here in this section collectively as "monomers") have one or more of the following properties: water soluble, partially macromeric character, containing hydrophilic groups, and being covalently reactive. When crosslinked to form gels, the resulting gels are tissue adhesive, elastic, and compliant. The monomers are preferably water soluble. Water soluble materials are soluble to at least about 0.1 gram per liter of a substantially aqueous solvent. A substantially aqueous solvent comprises at least about 50% by weight of water, and less than about 50% by weight of a non-aqueous, water-miscible solvent. If the polymers are not entirely water-soluble, they should be dispersible in water, and form micelles, typically with the aid of non-aqueous, water-miscible solvents. The non-aqueous solvent must be present in an amount that does not damage the tissue. Thus only a small amount of non-aqueous, water-miscible solvent should be present in the pre-gelled composition to minimize tissue irritation. Up to about 10% by weight of the solution can be a non-

aqueous, water-miscible solvent. Examples of non-aqueous, water-miscible solvents include ethanol, isopropanol, N-methylpyrrolidone, propylene glycol, glycerol, low molecular weight polyethylene glycol, DMSO, Benzyl alcohol, and benzyl benzoate. Liquid surfactants, such as poloxamers (e.g., PLURONIC™ surfactants) and some polyethylene glycol derivatives (e.g., some TWEEN™ surfactants) can also be used as non-aqueous, water-miscible solvents.

[0090] The monomers are preferably at least partially macromeric, and are more preferably substantially to completely macromeric. Macromers tend to be innocuous to tissue because they will not readily diffuse into or penetrate cells. A macromer is a reactive monomer consisting of a polymeric material with a number-average or weight-average molecular weight of about 500 Daltons or more and at least one reactive group. To form a crosslinked gel by chain-growth polymerization, the macromers, along with any other smaller monomers, in a solution must contain on average more than one reactive group (which may be a covalently reactive group, or a group that binds non-covalently to other macromers). For polymerizations involving step-growth polymerization, the macromers must contain on average more than two reactive groups, and the solution typically contain approximately equal numbers of the two different types of reactive groups. An example of step-growth polymerization is gelation by formation of urethane linkages from the reaction of isocyanate with the hydroxyl groups. For free-radical polymerization of unsaturated materials (chain-growth polymerization), the monomers must contain on average more than one reactive group to crosslink.

[0091] The monomers are preferably covalently reactive, and thus form a covalently crosslinked gel. The crosslinked gels are elastic, and further are both elastic and compliant with soft tissue at low polymer concentrations.

[0092] Any method of covalent polymerization is potentially useful in the formation of the gels. The reactive groups may include, without limitation, ethylenically unsaturated groups, isocyanates, hydroxyls and other urethane-forming

groups, epoxides or oxiranes, sulfhydryls, succinimides, maleimides, amines, thiols, carboxylic acids and activated carboxylgroups, sulfonic acids and phosphate groups. Ethylenically unsaturated groups include acrylates and other unsaturated carboxylic acids, vinylic and allylic groups, cinnamates, and styrenes. Activated carboxyl groups include anhydrides, carbonylimidazoles, succinimides, carbonyl nitrophenols, thioesters, O-acyl ureas, and other conjugated carbonyls. In general, any reactive group that will covalently bond to a second and that can maintain fluidity when exposed to water for enough time to allow deposition and reaction is of use in making a suitable reactive macromer. Due to their excellent stability and slow reactivity in aqueous solutions, ethylenically unsaturated reactive groups are preferred.

[0093] The polymerization reaction does not have to result in covalent bonds. A number of materials are known which can form gel structures by changing the ionic conditions of the medium (e.g. alginate) or by changing the temperature of the medium (e.g., agarose, certain poloxamers). Polysaccharides are typical of these materials. Gel-like structures can be formed from proteins, such as gelatin or fibrin. While it maybe more difficult to get these materials to adhere strongly to tissue, they are potentially of use in the hydrogels, particularly as depots for the drug.

[0094] Gel formation can be accelerated by inclusion of small (non-macromeric) polymerizable molecules that can assist in linking larger, polymeric macromers. These typically have molecular weights less than about 100 Da, more preferably less than 500 Da. For free radical polymerization, any of the common ethylenically unsaturated molecules can be used. These include derivatives of acrylic and methacrylic acid, such as acrylamide, hydroxyethyl methacrylate (HEMA), and diacrylated or polyacrylated glycols and oligoglycols. Allyl groups (e.g., allyl glycidyl ether) and vinyl groups (e.g., N-vinyl caprolactam and N-vinyl pyrrolidone) are also of use. Other unsaturated compounds include cinnamic acid and its esters, and maleic, fumaric and itaconic acids and their derivatives.

[0095] Polymerization is initiated by any convenient reaction, including photopolymerization, chemical or thermal free-radical polymerization, redox reactions, cationic polymerization, and chemical reaction of active groups (such as isocyanates, for example.) Polymerization is preferably initiated using photoinitiators. Photoinitiators that generate a free radical or a cation on exposure to UV light are well known to those of skill in the art. Free-radicals can also be formed in a relatively mild manner from photon absorption of certain dyes and chemical compounds. The polymerizable groups are preferably polymerizable by free radical polymerization. The preferred polymerizable groups are acrylates, diacrylates, oligoacrylates, methacrylates, dimethacrylates, oligomethacrylates, cinnamates, dicinnamates, oligocinnamates, and other biologically acceptable photopolymerizable groups.

[0096] These groups can be polymerized using photoinitiators that generate free radicals upon exposure to light, including UV (ultraviolet) and IR (infrared) light, preferably long-wavelength ultraviolet light (LWUV) or visible light. LWUV and visible light are preferred because they cause less damage to tissue and other biological materials than short-wave UV light. Useful photoinitiators are those which can be used to initiate polymerization of the macromers without cytotoxicity and within a short time frame, minutes at most and most preferably seconds. Exposure of dyes, preferably in combination with co-catalysts such as amine, to light, preferably visible or LWUV light, can generate free radicals. Light absorption by the dye causes the dye to assume a triplet state, and the triplet state subsequently reacts with the amine to form a free radical which initiates polymerization, either directly or via a suitable electron transfer reagent or co-catalyst, such as an amine. Polymerization can be initiated by irradiation with light at a wavelength of between about 200-1200 nm, most preferably in the long wavelength ultraviolet range or visible range, 320 nm or higher, and most preferably between about 365 and 550 nm. Numerous dyes can be used for photopolymerization. Suitable dyes are well known to those of skill in the art. Alternatively, suitable chemical, thermal and redox systems may initiate the polymerization of unsaturated groups by generation of free radicals in the initiator

molecules, followed by transfer of these free radicals to the unsaturated groups to initiate a chain reaction. Examples include but are not limited to peroxides, other peroxygen compounds, and azobisbutyronitrile.

[0097] As used herein, a "biodegradable" material is one that decomposes under normal in vivo physiological conditions into components that can be metabolized or excreted. Functional groups having degradable or resorbable linkages are incorporated into the structure of the hydrogel matrix to provide for its resorption over time. These functional groups may be incorporated within the macromers to form part of the backbone of the polymer strands of the hydrogel or as crosslinks between the polymer strands. Examples of degradable units may include, but are not limited to, esters, carbonates, carbamates and the like. The length of time it takes for the hydrogel to biodegrade may be tailored to provide a hydrogel that persists long enough to generate the required tissue level of the drug through the treatment period, which can last up to the seventh postoperative day, or preferably up to the tenth or fourteenth day. Given the achievement of this objective, shorter degradation or resorption times such as less than about three months are generally preferred. Degradation or resorption times less than about fifteen days are particularly preferred.

[0098] As used herein, a "biocompatible" material is one that stimulates only a mild, often transient, implantation response, as opposed to a severe or escalating response. Biocompatibility may be determined by histological examination of the implant site at various times after implantation. One sign of poor biocompatibility can be a severe, chronic, unresolved phagocytic response at the site. Another sign of poor biocompatibility can be necrosis or regression of tissue at the site. In the preferred embodiment, a biocompatible material elicits a minimal or no fibrosis or inflammation. This can be achieved through selection of hydrogel composition, and particularly through the use of hydrogel components resulting in degradation of the hydrogel in vivo in less than about two weeks, more preferably within seven to ten days.

[0099] In a preferred embodiment, the hydrogel composition is selected to provide acceptable levels of fibrosis or tissue reaction. This can be achieved through the selection of the reactive formulation, and other techniques known to those skilled in the art in drug delivery utilizing polymeric delivery devices.

[00100] Preferably, the inotropic agents are poorly soluble in water (i.e. hydrophobic). In terms of the solubility classification of the United States Pharmacopoeia (USP 24/NF 19, effective Jan. 1, 2000; p. 2254), the preferred solubility classes are: "slightly soluble", requiring 100 to 1000 parts of solvent to dissolve; "very slightly soluble", requiring 1000 to 10,000 parts of solvent; and "practically insoluble, or insoluble", requiring over 10,000 parts of solvent. Collectively, these classes are defined herein as "poorly soluble".

[00101] An inotropic agent applied in a single application directly to the heart is expected to be similarly or more effective to intravenous administration, with a potential reduction in side effects because a lower required dose and limited spread is anticipated.

[00102] The slow dissolution rate for poorly soluble inotropic agents controls their rate of efflux from the gel. The rate of efflux for such inotropic agents can also be controlled by selecting the particle size of the drug particles that are suspended in the macromer solution before its polymerization. Particles of a particular size can be made by any known method, including grinding, milling, cryofracture, precipitation, spraying, spray drying, and/or classification. Dispersion and stabilization of the particles within the macromer solution may be achieved with the use of surfactants.

[00103] When more soluble inotropic agents are used, their efflux rate from the gel can be altered to achieve the necessary delivery rate. Such soluble inotropic agents include those falling in United States Pharmacopoeia classes "very soluble", "freely soluble", "soluble", and "sparingly soluble". Typical means of altering release rates include encapsulating the agents in micro particles or liposomes and conjugating

the agents to macromolecules. They can be made less soluble by altering the salt or using the free acid/base form of the agents.

[00104] In one embodiment, pre-encapsulation is used for the small, water-soluble drugs (typically of molecular weights less than 1000 Da) that are incorporated into hydrogels, to decrease the rate of release of these drugs. The encapsulation may be by any conventional means. One means is entrapment in micro particles of a degradable, water-insoluble polymer. Typical materials are polymers and copolymers of lactic acid, glycolic acid, and copolymers thereof (e.g., PLGA). Other materials used to form suitable micro particles are copolymers of ethylene and vinyl acetate (EVAC) and polymers of anhydrides, such as poly sebacic anhydride. Particles of drug may also be pre-encapsulated with polymers such as EVAC and PLGA, or with thin layers of materials that dissolve in vivo, for example, the enteric coatings or other coatings typically used for oral delivery, such as gelatin.

[00105] Release of more soluble inotropic agents can be slowed by conjugating small molecules to polymers by degradable or reversible linkages. Many such systems are described in the art. In one embodiment, such systems are generated by immobilizing a binding or targeting molecule for the drug, such as an antibody or lectin, which is saturated with the drug, in the gel. In another typical embodiment, drug is attached to a polymer bearing reactive groups, such as to the hydroxyl of polyvinyl alcohol, to a carboxyl, sulfonate or amine group of a polysaccharide or the hydroxyl or carboxyl of an alpha-hydroxy acid (e.g., lactic or glycolic acid), or to a carboxylic group on a polymer (e.g., alginate, polyacrylic acid) via an anhydride, an ester, a carbonate, or carbamate linkage. Many similar methods are described in the art.

[00106] The solubility of some agents can be decreased by preparing them in their neutral ("free base") form. Such agents often can also be administered as suspensions in oil, which in turn is dispersed in water, usually with surfactant stabilizers.

[00107] The level of loading of the inotropic agent in the delivery vehicle will normally be as high as practical, while leaving a margin of loading to prevent premature precipitation or aggregation, or inhibition of gel formation. The concentration of the inotropic agent can be between 0.5 and 1% by weight, but this will depend in part upon the source and form of the inotropic agent. Gel polymerization rate and final gel may be significantly affected by drug concentration. Use of other macromers affects the optimal level. Fortunately, acceptable loading ranges are easily determined for a particular system by varying the loading and determining the properties of the formed gel.

[00108] In one method, the inotropic agent is provided in a formulation that forms a hydrogel in vivo, i.e. after its components are administered to the heart.

[00109] In a second method, the inotropic agent is provided to the patient in a preformed hydrogel "patch", i.e. formed before administration to the heart.

[00110] The hydrogels of the present invention are formed by a polymerization reaction, which may be any reaction that can be carried out in a substantially aqueous environment and is not damaging to tissue. The gels may be polymerized in vivo or in vitro.

[00111] The adherence of gels to tissue can be optimized by techniques that employ functional primers, as described in U.S. Pat. No. 5,800,373 to Melanson et al., U.S. Pat. Nos. 5,844,016, or 5,900,245 to Sawhney et al. for gels formed by polymerization of ethylenically unsaturated precursors. Suitable gel compositions form strong bonds to tissue. These techniques are also applicable to creating strong adherence of the materials to tissue, including tissue to which it is difficult to obtain adherence by conventional methods, for example, cartilage.

[00112] A general procedure for applying materials to the tissue involves brushing or dabbing primer over a larger area than that over which the material is applied. Thereafter, material is brushed or dabbed over the deposited primer. Then

bulk material is applied by dripping (if liquid) or spreading (if paste) over yet a smaller area of the treated zone. Then light (at appropriate wavelength, intensity, distance and for an appropriate time) is applied at each zone, or other means of polymerizing the material are used.

[00113] Methods for in vivo and in vitro hydrogel polymerization are known in the art, for example as described in published patent applications 20020150622 and 20050004428, which are hereby incorporated by reference.

[00114] For in vivo polymerization, the inotropic agent is formulated in appropriate excipients (if any) in a vial, and is taken up in a known amount of hydrogel forming material. This solution is applied to the tissue, and polymerization is effected to form a gel adherent to the tissue. Preferably, the solution is polymerized by illumination of a photoinitiator or photosensitizer in the solution. In this case, the mixing of two solutions at the time of application will not necessarily form a gel; however once the solutions are illuminated by light of an appropriate frequency, a gel will form, as described in U.S. Pat. No. 5,410,016 to Hubbell et al. incorporated herein by reference in its entirety.

[00115] In vivo polymerization has the advantage of being able to produce "good" to "excellent" adherence when polymerized on the tissue surface. This is particularly true when the tissue is first primed or otherwise pretreated with an agent (primer) stimulating polymerization (as known to those skilled in the art, for example, as described in U.S. Pat. No. 5,844,016 to Sawhney et al. and U.S. Pat. No. 5,834,274 to Hubbell et al. incorporated herein by reference in their entirety) prior to the application of the macromer composition containing the inotropic drug. See also U.S. Pat. Nos. 5,567,435; 5,844,016; 5,986,043; 6,060,582; and 6,306,922 incorporated herein by reference in their entirety. In these methods, an aqueous solution containing a photoinitiation system, including one or more photoinitiators, photosensitizers and co-initiators, amine or amide electron transfer agent, redox accelerant system for the photoinitiation system (such as a metal ion and a peroxide); and a photopolymerizable

macromer solution, are applied to the tissue, and the solution is polymerized by exposure to UV or visible light at room or body temperature.

[00116] For in vitro polymerization, hydrogel patches containing the inotropic agent are polymerized in vitro and then adhered to the surface of the heart. The inotropic agent in any suitable formulation can be entrapped in a hydrogel in vitro, which is optionally preserved by freezing or drying, and is subsequently transferred to the cardiac tissue. The preformed gel patch, or more than one preformed gel patch, is then adhered to the cardiac tissue. Adhesion of the patch may be achieved by the polymerization of a hydrogel-forming material, which may be the same as or different from the material used to form the gel patch, placed between the preformed gel patch and the tissue, or optionally encapsulates the entire pre-formed gel. Adhesion may also be achieved by completing polymerization of a partially polymerized gel patch onto the tissue. A partially polymerized gel patch is prepared by reducing time exposure to polymerization conditions or by quenching polymerization.

[00117] In vitro polymerization has the advantage of providing a reliable means of delivering a precisely defined dose of the inotropic agent. The preformed gels should have the same properties as gels formed in vivo. This method of application may be regarded as another form of application of an encapsulated drug to the tissue, since the adhesion to the tissue is provided by a hydrogel that is formed in situ on the tissue. The preferred method of attaching the gels to the tissue surface is to use macromer solutions to adhere the preformed gel to the tissue. Adherence is also preferably in the "good" to "excellent" range.

[00118] A material is tissue adherent if it requires a force to remove the material from the tissue. Thus, the general and practically useful measurement of adherence is that the gel, when applied to the tissue, remains attached to the tissue for at least as long as is required to obtain the therapeutic effect of the drug. Typically, this time period will be sufficiently long to observe at least about 10% elution of the

drug, and preferably 20% elution or more, before detachment or degradation of the gel.

[00119] Ex vivo tests can be used to determine a material's potential adherence. In evaluating potential adherence of materials, it is useful to have an in vitro test to determine formulations that are likely to have the desired degree of adherence to the tissue surface. One method of judging adherence is to require that upon a gradual increase in a detaching force, the force required to remove the gel from the tissue is greater than or approximately equal to the force required to cause cohesive failure of the gel (or the tissue, if lesser). Thus on attempting to remove the material, either the material or the tissue experiences cohesive failure at a lesser force than, or at approximately the same force as, the force at which the bond between the material and the tissue experiences adhesive failure. Materials that require a force of about 20 dynes/cm^2 to remove them from the tissue are sufficiently adhesive for delivery of inotropic agents.

[00120] Adherence can be described qualitatively as "excellent", when cohesive failure is required for removal from the surface, "good" when failure is partially cohesive and partially adhesive, "fair" when removal requires only adhesive failure (i.e., detachment of the gel from the surface) and more than 20 dynes/cm^2 of force is required to produce adhesive failure, and "poor" if none of these criteria are satisfied. Force can be measured using a mechanical properties tester, such as an Instron™ tester or other device.

[00121] The delivery vehicles of the invention are preferably highly compliant with the tissue to which they adhere. Thus, the delivery vehicles stretch and bend along with the tissue. Cardiac tissue is in continual motion, and the delivery vehicle should not significantly disturb this motion. It is preferable that the response to stress within these limits be substantially elastic, i.e., reversible. Thus the delivery vehicle should remain as a coherent material for at least the period required for delivery of the inotropic agent.

[00122] Techniques for producing strong adherence of a preformed hydrogel, a patch, or other delivery vehicle to the cardiac tissue include applying an initiator or promoter of polymerization to the tissue at the site; applying a thin layer of gelling solution having a high concentration of a polymerizable reagent at the site; applying materials bearing one half of a reactive pair to the site, optionally a member of a reactive pair which is also reactive with tissue; and applying mechanical action to a layer of polymerizable material on the tissue (before polymerization) to ensure that no layer of fluid, such as mucus or the like, separates the polymerizable material from the tissue.

[00123] As described herein, the delivery vehicles of the invention, including hydrogels, patches, ointments and creams, can be applied at the time of surgery and the drug delivered directly to the affected cardiac tissue. For a hydrogel polymerized in situ, the gel can be applied in open surgery by any method. In one embodiment, the delivery vehicle such as an ointment, cream, or gel is preferably brushed or sprayed onto the tissue surface for example by using a device designed for percutaneous use, but may be dripped from a mixing apparatus.

[00124] The therapeutic compositions of this invention are administered by local administration to the heart, as by application of a patch, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[00125] It is important to provide a way for the physician to deliver a well-defined amount of the inotropic agent, so that the therapeutic effect can be obtained.

[00126] The dosage of the inotropic agents for use in a human or animal and the minimum duration can be determined with only routine experimentation in view of animal studies and the known drug kinetics, including half-life, solubility and other