Modification Form for Permit BIO-UWO-0257

Permit Holder: Alp Sener

Approved Personnel
(Please stroke out any personnel to be removed)

Amy Mok

Additional Personnel
(Please list additional personnel here)

Ian Lobb
Omar Champsi
Justin Zhu
Mike Davison
Joshua Hwang

	Please stroke out any approved Biological Agent(s) to be removed	Write additional Biological Agent(s) for approval below. Give the full name
Approved Microorganisms		
Approved Primary and Established Cells	Human [primary] CD4 T-cells from spleen Rodent [primary] CD 4 T cells from spleen and kidney. Human [established] HUV-EC-C, PT2 cells.	Hep 3B 2.1-7 [Hep 3B] cells contain HEPATITIS B Homo sapiens
Approved Use of Human Source Material	Human blood (whole) or other body fluid, Human blood (fraction or other body fluid, human organs or tissues (unpreserved).	
Approved Genetic Modifications (Plasmids/Vectors)		
Approved Use of Animals		
Approved Biological Toxin(s)		
Approved Gene Therapy		
Approved Plants and Insects		

PLEASE ATTACH A MASTER LIST OF ALL BIOLOGICAL AGENTS.
PLEASE ATTACH A BRIEF RESEARCH PROTOCOL OF AGENTS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF.

As the Principal Investigator, I have ensured that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/newposition.htm

Signature of Permit Holder:

Current Classification: 2 **Containment Level for Added Biohazards:** HEP 3B cells

Date of Last Biohazardous Agents Registry Form: Aug 19, 2010

Date of Last Modification (if applicable):

BioSafety Officer(s):

Chair, Biohazards Subcommittee: **Date:** Nov 16th 2011

BRIEF OVERVIEW

Anemia of end stage renal disease (ESRD) is a tremendous concern both for patients and health care providers alike. This condition affects over 90% of all hemodialysis patients and is associated with numerous physiological comorbidities. One potential etiology is the lack of erythropoietin (Epo) production from the kidney. Amongst available treatment strategies, erythropoiesis-stimulating agents (ESA) are considered to be the most optimal therapy available, however their clinical efficacy is variable. Investigation into the lack of Epo production in ESRD suggests that hypoxia induced release of the small molecule nitric oxide (NO) and subsequent rise in cGMP may act to decrease GATA-2 mRNA expression and increase Epo production by negatively affecting the GATA element in the Epo promoter in Hep3B cell lines (a known platform to study human Epo gene activation)3. The recent discovery of hydrogen sulphide (H2S) as the third endogenous gasotransmitter has led to its implication in a variety of physiological and pathophysiological processes. Most recently, H2S has been shown to play an increasingly significant role in renal oxygen sensing capability, especially during periods of low oxygen tension. Although ESRD is known to create a state of renal hypoxia and that urinary H2S levels have been shown to be diminished in ESRD patients on hemodialysis, the link between ESRD-induced low intrarenal H2S production and diminished Epo production is unknown, and may provide a novel insight to anemia of chronic kidney disease. The objective of the current study is to determine whether H2S plays a role in hypoxia induced Epo production in Hep3B cell lines. We hypothesize that the inhibition on H2S production will lead to a decline in Epo production and that this effect is independent of nitric oxide signaling pathways.
Our findings may pave the way for various therapeutic strategies against anemia of ESRD which could include the use of novel long-acting, orally bioavailable, H2S releasing molecules that are currently under development.

EXPERIMENTAL PROTOCOL
The Hep3B cell line will be obtained from the American Type Tissue Culture Collection (Rockville, MD, USA). Cells will be cultured in DMEM, supplemented with penicillin (100 U/mL), streptomycin (100 μ/mL) and 10% heat-inactivated FBS in 10 cm dishes. Cells will be maintained in a humidified 5% CO2/95% air incubator at 37°C. The cells will be grown under conditions of hypoxia (1% oxygen) or normoxia as previously described for 24 hours3. The cells will be treated with/without the inhibitor of CSE (dL-propargylglycine: PAG, 1mM) as well as the inhibitor of nitric oxide synthase L-NMMA (1000μM) in the culture medium. Briefly, after incubation under hypoxic/nor- moxic and/or PAG-treated/untreated conditions, the cells will be harvested and cellular extracts prepared. In certain instances, cells will also be treated with the H2S donor (NaHS)
at 50μM, 200μM and 400μM quantities or the NO precursor, L-arginine (10μM). We currently use 200μM NaHS in our in vitro assays for ischemia reperfusion injury and find that it is cytoprotective. Total cellular RNA will be prepared by conventional methods and total GATA-2 mRNA expression will be quantitated using RT-PCT techniques (the oligonucleotide primers for human GATA-2: upstream, 5'-CCC TAA GCA GCG CAG CAA GAC-3', and downstream, 5'-GAT GAG TGG TCG GTT CTG GCC–3'), and total cGMP (measure of NO activity) and Epo production quantitated using ELISA assays.

We will use the reporter plasmid pEPLuc described by Imagawa et al3 as a basic plasmid construct, in which both the 126-bp 3' Epo enhancer [120 to 245-bp 3' of the poly(A) addition site] and the 144-bp minimal Epo promoter (from -118 to +26 relative to the transcription initiation site) will be placed upstream of the firefly luciferase (Luc) gene in pXP2, resulting in Pwt or V2-Ewt-Pwt-pXP2. This enhancer contains a hypoxia-inducible factor 1 (HIF-1) binding site and steroid receptor response element. A total of 6 to 7x105 cells in six-well tissue culture plates will be washed with serum free media. A mixture containing lipofectin (20μg/ well), DNA constructs (2μg/well) and beta-galactosidase (1 μg/well) as an internal standard will be co-transfected and the plates were incubated for 12 hours. The media will then be changed to DMEM/FCS with or without PAG/L-NMMA/NaHS and incubated for 24 hours under hypoxic/normoxic conditions. Luc activity in 20 μL of the cell extract will be determined by an Autolumat luminometer for 10 seconds. Each measurement of relative light units will be corrected by subtraction of the background and standardized to the beta-galactosidase internal transfection control activity. Hypoxic inducibility will be defined as the ratio of the corrected relative light units of the hypoxic: normoxic dish.

Cell Biology

ATCC® Number:	**HB-8064™** Order this Item	Price:	**$279.00**

Designations:	Hep 3B2.1-7 [Hep 3B, Hep-3B, Hep3B]
Depositors:	Wistar Institute
Biosafety Level:	2 [Cells Contain HEPATITIS B]
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens*
Morphology:	epithelial
Source:	**Organ:** liver **Disease:** hepatocellular carcinoma
Cellular Products:	alpha fetoprotein (alpha-fetoprotein); hepatitis B surface antigen (HBsAg); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; [3525] alpha1 antichymotrypsin (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; complement (C3, C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); [3525] alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein); Gc globulin [3525]
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (Roche Transfection Reagents)
Tumorigenic:	Yes
DNA Profile (STR):	Amelogenin: X CSF1PO: 8 D13S317: 12,14 D16S539: 10 D5S818: 13 D7S820: 8,10 THO1: 6,7 TPOX: 9 vWA: 17
Cytogenetic Analysis:	modal number = 60 with a subtetraploid mode of 82; has a rearranged chromosome 1 [3525]
Age:	8 years juvenile
Gender:	male
Ethnicity:	Black

Related Links ▸

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Login Required ▸

Product Information Sheet

Comments:	This line contains an integrated hepatitis B virus genome. [1205] [3525]
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5%
Subculturing:	**Protocol:** Remove medium, and rinse with 0.25% trypsin, 0.53 mM EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks. **Subcultivation Ratio:** A subcultivation ratio of 1:4 to 1:6 is recommended **Medium Renewal:** Twice per week
Preservation:	**Freeze medium:** Complete growth medium, 95%; DMSO, 5% **Storage temperature:** liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2003 recommended serum:ATCC 30-2020
References:	1205: Knowles BB, et al. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209: 497-499, 1980. PubMed: 6248960 3525: Knowles BB, Aden DP. Human hepatoma derived cell line, process for preparation thereof, and uses therefor. US Patent 4,393,133 dated Jul 12 1983 22909: Aden DP, et al. Controlled synthesis of HBsAg in a differentiated human liver carcinoma- derived cell line. Nature 282: 615-616, 1979. PubMed: 233137 24388: Darlington GJ, et al. Growth and hepatospecific gene expression of human hepatoma cells in a defined medium. In Vitro Cell. Dev. Biol. 23: 349-354, 1987. PubMed: 3034851

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THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: September 25, 2009
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR	Dr. Alp Sener
SIGNATURE	
DEPARTMENT	Surgery
ADDRESS	LHSC U-UH C4-208 Wiadermere Rd.
PHONE NUMBER	519-663-3352
EMERGENCY PHONE NUMBER(S)	519-630-5921
EMAIL	~~sener@uhp~~ alp.sener@lhsc.on.ca

Location of experimental work to be carried out: Building(s) SDR1 Room(s) 2.30

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 12.0, Approvals).

FUNDING AGENCY/AGENCIES: Canadian Urological Association Scholarship Fund
GRANT TITLE(S): Title: Role of hydrogen sulphide in mitigating renal ischemia-reperfusion injury. American Society of Transplant Surgeons/Novartis and CIHR-IRF 2010. Effect of Thymoglobulin on T-lymphocyte subset reconstitution and Function following renal transplantation.

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED.

Names of all personnel working under Principal Investigators supervision in this location:

Amy Mok

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? O YES ⓞ NO
(including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? O YES O NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ⓞ YES O NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	ⓞ Yes O No	CD4 T cells from Spleen AS	Not applicable
Rodent	ⓞ Yes O No	CD4 T cells from spleen and robot kidneys	2009-076 Server
Non-human primate	O Yes ⓞ No	—	—
Other (specify)	O Yes ⓞ No	—	—

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	O Yes O No	{HUV-EC-C {PT2 cells	ATCC Jevnikar's lab
Rodent	O Yes O No		
Non-human primate	O Yes O No	—	—
Other (specify)	O Yes O No	—	—

Aug 12/10 ?A.M. $ for A.S.

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ⊘1 ⊘2 O 3
rodent human.

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ⊘YES O NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid	LHSC, Transplant program.	O Yes ⊘No O Unknown	N/A	O 1 ⊘2 O 3
Human Blood (fraction) or other Body Fluid	"	O Yes ⊘No O Unknown	N/A	O 1 ⊘2 O 3
Human Organs or Tissues (unpreserved)	"	O Yes ⊘No O Unknown	N/A	O 1 ⊘2 O 3
Human Organs or Tissues (preserved)	—	Not Applicable	—	Not Applicable

* tested for EBV, CMV, HIV, HTLV 1/2, HCV, HBV, RPR, VDRL

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? O YES ⊘NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? O YES, complete table below O NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results

* Please attach a Material Data Sheet or equivalent if available.

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below O NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ O NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ O NO
- SV 40 Large T antigen O YES O NO
- E1A oncogene O YES O NO
- Known oncogenes O YES, please specify _____ O NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ O NO

4.5 Will virus be replication defective? O YES O NO

4.6 Will virus be infectious to humans or animals? O YES O NO

4.7 Will this be expected to increase the containment level required? O YES O NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES ~~O NO~~
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: _____
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? O YES ~~O NO~~ If no, please proceed to section 7.0

6.2 Name of animal species to be used_____

6.3 AUS protocol # _____

6.4 Will any of the agents listed in section 4.0 be used in live animals O YES, specify: _____ O NO

6.5 Will the agent(s) be shed by the animal: O YES O NO, please justify:

_____

_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES ⊘NO
- Pound source cats O YES ⊘NO
- Cattle, sheep or goats O YES ⊘NO
- Non-human primates O YES, please specify species _____ ⊘NO
- Wild caught animals O YES, please specify species & colony # _____ ⊘NO
- Birds O YES ⊘NO
- Others (wild or domestic) O YES, please specify _____ ⊘NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? O YES ⊘NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s)_____
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin_____

8.4 How much of the toxin is handled at one time*? _____

8.5 How much of the toxin is stored*? _____

8.6 Will any biological toxins be used in live animals? O YES, Please provide detais:_____ O NO

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES ⊘NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:
_____
_____
_____

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions:
_____
_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O YES ⊙ NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 ⊙ NO

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES O NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE _____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

13.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 ① 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?

O YES, permit # if on-campus_____
① NO, please certify
O NOT REQUIRED for Level 1 containment

SDR1 231A inspected June 29/10 (shared with Dr. Gunaratnam) ak.

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE _____ Date: March 12, 2010.

14.2 Please describe additional risk reduction measures will be taken beyond containment level 1, 2, or 3 measures, that are unique to this agent.

None.

14.3 Please outline what will be done if there is an exposure to the biohazards listed, such as a needlestick injury:

see email. attached

Any exposure to human blood via skin puncture will be reported to the Biohazards committee and the person will be directed to the E.R.

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: Susan Koval
Date: August 19, 2010

Safety Officer for Institution where experiments will take place: SIGNATURE: Mike Ryder (Lawson)
Date: MAR. 29/10

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: J Stanley
Date: Aug 13/10

Approval Number: BIO-UWO-0257 Expiry Date (3 years from Approval): Aug 18 2013

Special Conditions of Approval:

Subject: Re: Biohazardous Agents Registry Form: Sener
From: Alp Sener <Alp.Sener@lhsc.on.ca>
Date: Tue, 10 Aug 2010 15:22:12 -0400
To: Jennifer Stanley <jstanle2@uwo.ca>
CC: Amy Mok <Amy.Mok@lhsc.on.ca>, "Amy Y. Mok" <amok@uwo.ca>

HI Jennifer,

Thank you for your email. Yes, if there is a needlestick injury or other exposure to biohazardous materials, we will immediately contact and visit Workplace Health.

Thanks again
Alp

14.3

Alp Sener, MD, PhD, FRCSC
Assistant Professor of Surgery & Microbiology and Immunology
Director, Renal Transplant Fellowship Program
Schulich Clinician Scientist, University of Western Ontario
LHSC-University Hospital
C4-208, 339 Windermere Road
London, Ontario, Canada N6A 5A5
Tel: 519-663-3352
Fax: 519-663-3858
alp.sener@lhsc.on.ca

Jennifer Stanley <jstanle2@uwo.ca> 2010/08/10 03:09 PM >>>

Hi Dr. Sener

Thanks for your recent submission. I understand that this work is now being done in SDRI.

Please confirm that in case of a needlestick or other exposure, your lab workers will visit Workplace Health (please see pages 26-28 of the Biosafety Manual for more information).
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/biosafety manual.pdf

Regards,
Jennifer

-------- Original Message --------
Subject: Fwd: Re: Fwd: Biohazardous Agents Registry Form: Sener
Date: Fri, 25 Jun 2010 12:08:03 -0400
From: Alp Sener <Alp.Sener@lhsc.on.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>
CC: Amy Mok <Amy.Mok@lhsc.on.ca>

Hi Jennifer,

Sorry, I thought that you wanted a list of chemicals, my apologies.

The biohazards we have in the lab are as follows:

1) HUVEC (human umbilical vein endothelial cells): used for tissue culture and experiments. They are stored/cycled in the 37C incubator. Contact with the cells is always done under the Biosafety hood using appropriate gloves and lab coat under sterile conditions. We will have a stock population stored in the -80 freezer in the upcoming months.

2) Murine renal tubular epithelial cells: used for tissue culture and experiments. They are stored/cycled in the 37C incubator. Contact with the cells is always done under the Biosafety hood using appropriate gloves and lab coat under sterile conditions. We will have a stock population stored in the -80 freezer in the upcoming months.

3) Human serum and splenocytes. These will be obtained from renal transplant recipients and organ donors, respectively by LHSC. We will store 1mL of serum from each renal recipient in the -80C freezer for archival purposes. The rest of the serum will be processed within 24h of procurement in the main lab. Experiments will involve cell sorting for T-lymphoctes and antibody labeling for analysis of syurface phenotypic markers on the flow cytometer in LHRI. Once the data is accrued, the samples will be disposed of in biohazard safety containers. Gloves, eye shields and lab coats will be worn at all times.

I hope that this helps out. If you need further information, please let me know. Unfortunately I will not be present for the lab inspection next week as we are scheduled to have a bay on Tuesday. If there are any concerns, please let me know.

Best regards
Alp

Alp Sener, MD, PhD, FRCSC
Assistant Professor of Surgery & Microbiology and Immunology
Director, Renal Transplant Fellowship Program
Schulich Clinician Scientist, University of Western Ontario
LHSC-University Hospital
C4-208, 339 Windermere Road
London, Ontario, Canada N6A 5A5
Tel: 519-663-3352
Fax: 519-663-3859
alp.sener@lhsc.on.ca

Subject: Re: Biohazardous Agents Registry Form: Sener
From: Alp Sener <Alp.Sener@lhsc.on.ca>
Date: Sat, 03 Apr 2010 00:46:17 -0400
To: jstanle2@uwo.ca

Dear Jennifer,

Thank you for your email. The work is being done at SDRI but when we first started, we were in Tony Jevnikar's lab in Lawson.

I hope this helps.

Thanks
Alp

Alp Sener, MD, PhD, FRCSC
Assistant Professor of Surgery & Microbiology and Immunology
Director, Renal Transplant Fellowship Program
Schulich Clinician Scientist
University of Western Ontario
London Health Sciences Center
UH C4-208, 339 Windermere Road
London, Ontario, Canada N6A 5A5
Tel: 519-663-3352
Fax: 519-663-3858
E-mail: alp.sener@lhsc.on.ca

Jennifer Stanley <jstanle2@uwo.ca> 04/01/10 3:56 PM >>>

Hi Dr. Sener
I received your form today - thanks. There are some items missing so I have sent it back to you via the mail.
I am a bit confused why Gail Ryder, from LHRI, signed it as the "Safety Officer for Insitution where experiments will take place". Is there some research going on at LHRI? According to the first page the location for the work is SDRI 230??? I guess the question is, where is the work being done?
Thanks
Jennifer

Fall 2009 Competition Application – Deadline Midnight on October 30, 2009

Please Refer to New Guidelines BEFORE Completing Application!

1. Principal Investigator's Name, and campus mailing address and phone:

Alp Sener MD, PhD
Department of Surgery, C4-208, UH-LHSC
339 Windermere Rd, London, ON N6A 5A5
Tel: 519-663-3352

Indicate the Department of your Main Appointment or Other Lawson Affiliation :

Surgery

Check where the research will be carried out:

LHSC **X** St. Joseph's ☐ Western ☐

Date of Original Appointment to LHSC, St. Joseph's and/or Lawson:

July 1, 2009

Date of First Appointment to The University of Western Ontario:

July 1, 2009

Lawson Research Theme Affiliation:

Transplantation & Immunology

Is this research project being resubmitted?

Yes ☐ **No** X

Co-Investigator(s) Name(s) and Lawson affiliation:

i) Collaborators only (Drs. Anthony Jevnikar and Patrick Luke)
ii)
iii)
iv)

2. Title of Project:

Effect of Thymoglobulin on T-lymphocyte Subset Reconstitution and Function Following Renal Transplantation

3. Application for Graduate Student Support: (Type 'X' in the appropriate box)

☐ YES X NO Name: Not applicable

4. Period of Support: Two Years Start/End Dates

January 1, 2010 to December 31, 2011

5. Total Amount Requested: $15,000

From Completed Application Budget Form

6. Has this project been submitted for review by the UWO Health Sciences Research Ethics Board?

☐ No, not applicable (no human subjects) ☐ Yes, approved (Attach Copy of Approval REB Notice)

X Yes, approval pending ☐ Not yet submitted

BACKGROUND AND RATIONALE FOR PROPOSED STUDY

The rise in the incidence of end stage renal disease (ESRD) is both a national and international concern. Worldwide, 1.8 million people were treated for ESRD in 2004--a 6% increase from 2003 and a 20% increase compared to 2000[1]. Although several forms of renal replacement therapy currently exist, renal transplantation provides the greatest potential for increased longevity and enhanced quality of life versus dialysis[2]. Unfortunately there is a rising discrepancy between the availability of donors and the exponential growth in patients on the transplant waiting list, with 5% of those patients dying each year while waiting for life saving organs[3]. In order to compensate for this discrepancy, the transplant community has made considerable efforts to expand the availability of donor organs through patient and public education campaigns, introduction of novel surgical techniques[4], as well as through the use of expanded criteria donor (ECD: donor age >60 years, or age 50-59 years with at least two comorbidities including hypertension, cerebrovascular death or serum creatinine at procurement >133 μmol/L) and donation after cardiac death (DCD) kidneys. The latter two have led to a considerable rise in the number of potential donors and currently makes up for approximately 25% of all transplants done in both the United States and Canada[5;6;7].

Our attempts at maximizing the use of available ECD and DCD kidneys comes at a price with a higher risk of delayed graft function and graft loss compared to kidneys from standard criteria donors[5]. Up to 30% of recipients of ECD and DCD kidneys lose their renal grafts within 5 years and up to 50% in 10 years[3]. This results in almost 25% of those patients going back onto renal replacement therapies and then becoming relisted for transplantation. In years to come, we will see a surge in this population of previously sensitized individuals (either from multiple previous transplants or from numerous blood transfusions while waiting on hemodialysis) who are going to be of higher immunologic risk. This higher immunological risk has been shown to translate into higher rates of acute T-cell mediated rejection, earlier graft loss and chronic allograft nephropathy[8].

The currently accepted strategy to best limit acute T-cell mediated rejection with this combination of donor/recipient variables has been to use a polyclonal T-cell depleting induction agent--the most common one being anti-thymocyte globulin (ATG). Unfortunately, despite the use of this potent drug, acute rejection continues to be a major obstacle to graft survival, especially in the previously sensitized population. The etiology behind this T-cell mediated rejection in the face of effective immunosuppression is not well understood. Accordingly, several key areas in the field of renal transplantation have recently been highlighted as requiring further investigation[9]. The development of an improved understanding of the mechanisms behind T-cell mediated rejection stands as one of the highlighted areas in need for further research and forms the basis of the current application.

In preliminary experiments, we used a novel mouse model with a trackable population of alloreactive memory CD4 T-cells[10] to determine their *in vivo* susceptibility to two T-cell depleting agents: anti-lymphocyte serum (ALS), as a generic T-cell depleting agent, and the more clinically relevant mouse ATG (mATG), and evaluated whether peripheral naive, memory and/or regulatory T-cells could reconstitute the depleted hosts. In summary, we found that memory T-cells are readily depleted with by mATG or ALS, and that *both* memory and naive CD4 T-cells undergo post-depletional homeostatic proliferation (Figure 1). We further described that naive T-cells exhibited *greater* homeostatic expansion than their memory counterparts, particularly in thymectomized hosts, and that these cells also underwent the most dramatic increase in proliferative turnover relative to steady state levels following mATG (Figure 2). Our findings also provided conclusive evidence that T-cell depletional agents such as mATG result in the conversion of previously quiescent naive T-cells to memory-phenotype cells, suggesting a potential mechanism for post-induction immune alterations[11]. As growing numbers of patients on the transplant waiting list are highly sensitized from previously failed transplants, our study has wide implications. The proposed translational studies will provide novel information which will ameliorate immunosuppressive protocols in the peri-transplant period to ultimately improve both short and long-term allograft and patient survival.

OBJECTIVES AND HYPOTHESES

Objective I: To determine the frequency, phenotype and degree of proliferation of newly reconstituted T-cells following Thymoglobulin induced T-cell depletion in both low- and high-immunological risk renal transplant recipients over time.

Hypothesis I: Following T-cell depletion with Thymoglobulin, the homeostatic proliferation of naive CD4 T-cells will be more pronounced than that observed in the memory or regulatory T cell population in low-immunological risk patients but will be equivalent in the high-risk group.

Objective II: To establish the *in vitro* recall responses to foreign antigen as measured by cytokine production by naive and memory CD4 T cells at various time points following Thymoglobulin-induced lymphodepletion in low- and high-immunological risk renal transplant recipients.

Hypothesis II: The newly reconstituted naive CD4 T-cells will possess greater alloreactivity to foreign antigen compared to their memory cohort in high-immunological risk but not in low-immunological risk renal transplant recipients over time.

PROPOSED RESEARCH

Patients will be enrolled in accordance with the policies and procedures established by the University of Western Ontario, Research Ethics Board (Application Pending Approval). All patients with chronic kidney disease presenting for renal transplantation to the London Health Sciences Center (LHSC) (both live renal transplant recipients and deceased donor renal transplant recipients) will be given the opportunity to participate in this study. Inclusion criteria will be those who have provided signed informed consent and do not have any exclusion criteria. Exclusion criteria include patients younger than 18 years of age and those who can not give informed consent. Patient demographics as well as baseline and subsequent clinic visit laboratory parameters will be collected.

Peri-operative management of each patient will be directed by the patient's clinical course in hospital. The study group will consist of two sub-populations. The first sub-group will include patients at high-immunologic risk for acute rejection (n=15: peak PRA level >40%, history of multiple blood transfusions, African-Americans, ECD/DCD donors, cold ischemic times >24 hours and re-transplants) and will therefore receive ATG induction as outlined by our immunosuppression protocol. The second sub-group will include low risk patients (n=15: peak PRA <40% and *de novo* transplants) who will also receive ATG induction. Patients will not be randomized into one treatment arm or another and will solely be selected according to the guidelines listed above. A control group will consist of transplant recipients who receive the non-depleting induction agent, Basiliximab (n=15). Since this agent does not influence T-cell kinetics, it is the ideal candidate for a control arm. Maintenance immunosuppression will be carried out according to the institutional immunosuppression protocols and will include a combination of tacrolimus, mycophenolate mofetil, and steroids.

Baseline blood samples (15 mL) will be obtained into heparinized tubes prior to ATG or Basiliximab administration at the time of the surgery as well as at early (2, 7, 14, 28 days post-transplant) and late (every month for 6 months following transplantation) time points to assess the reconstitution of T cell subsets. These samples will be obtained to coincide with routine and regularly schedules clinic appointments and blood draws.

Objective I: Determine the frequency, phenotype and proliferation of reconstituted T-cells after ATG

Peripheral blood leukocytes (PBL) will be isolated from red blood cells by density-centrifugation through ficoll and stained with fluorescently-tagged antibodies specific for CD3, CD4, and CD8, and counterstained with markers associated with a naive or memory phenotype including CD45RO, CD45RA and CD62L, the activation markers CD25 and HLA-DR, and receptors for key homeostatic cytokines such as IL-7 after manual sorting as previously described[10] and analyzed using a flow cytometer. Memory T cells will be distinguished from naive cells by expression of CD45RO (versus CD45RA in naive cells), and central from effector memory by co-expression of the lymph-node homing receptor CD62L[12]. If the patients undergo antibody-mediated rejection based upon renal biopsy pathology, we will also stain for B cells using CD20; these patients will then be excluded from continuing with the remainder of the study. Proliferation of the various T-cell subtypes will be assessed by staining for Ki67[13]. PBLs will be isolated as above, surface stained, fixed, and intracellularly stained with fluorescently-labeled anti-Ki67 antibody. Percentages of proliferating lymphocyte subsets, as defined by expression of Ki67, will be determined by flow cytometry. T_{regs} comprise a proportion of T cells exhibiting a $CD4^+CD25^+$ surface phenotype. Since activated CD4 T cells also express CD25 and are indistinguishable from T_{regs} based on surface phenotype alone, we will also stain for FoxP3 as previously described[14]. PBLs will be surface stained, fixed, and stained intra-cellularly with fluorescently-conjugated anti-FoxP3 antibody. The percentages of $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^-FoxP3^+$ T cells will be quantified using flow cytometry.

Objective II: Determine the *in vitro* alloreactivity of naive and memory CD4 T cells following ATG

The effects of Thymoglobulin or its control, Basiliximab, on the frequency of donor-specific alloreactive memory CD4 T cells in these patients will be assessed *in vitro*. We will stimulate recipient PBLs with donor APCs to assess IFN-γ and IL-2 production by donor-specific memory as well as naive CD4 T-cells using a 24-hour enzyme-linked immunospot (ELISPOT) assay and plate-reader, as previously described[11]. A higher frequency of cytokine-producing cells will indicate a greater percentage of alloreactive memory T cells or of naive cells acquiring a memory phenotype. We will monitor this donor-specific allo-response prior to Thymoglobulin or Basiliximab induction, as well as at 1 week, 28 days and 6 months after transplantation. If applicable, ELISPOT will be done prior to rescue therapy for acute T-cell mediated rejection and exclude these patients from continuing with the remainder of the study. The ability to perform this experiment requires a continuous supply of donor-derived APCs, which will be obtained by storing the donor spleen at the time of kidney procurement.

POTENTIAL LIMITATIONS

We may endure include difficulty with accruing adequate number of patients in each subgroup. However, our center transplants between 82-95 kidneys per year with approximately 25% being in the high-immunological risk category as defined in the current proposal. Confounders in the heterogeneity of the transplant recipient population, especially since patients will not be randomized into the various subgroups, will be handled using statistical methods. Further limitations include the possibility that we will not have an adequate number of PBLs for culture in addition to those needed for phenotypic studies. Additional blood samples may be needed during time-points for which ELISPOTs will be performed and will be coordinated with the clinic blood draws. Lastly, although we plan to analyze the peripheral lymphocyte population for 6 months after transplantation to assess the reconstitution of the overall T lymphocyte pool, should lymphocyte counts remain low and reconstitution incomplete after 6 months post-transplant, it may be necessary to continue peripheral blood monitoring for an extended period of time.

CANADIAN UROLOGICAL ASSOCIATION SCHOLARSHIP AWARD
RESEARCH PROPOSAL

Statement of Objectives

The nature of transplantation leads to tissue injury as organs are damaged by the loss of blood supply and ischemia associated with the procurement procedure. The potential benefit of donor tissue and storage modification to protect organs has not been intensively investigated as mainstream approaches to improving transplant survival remains focused on pharmacological inhibition of immune cell activation. The research group that I will be primarily collaborating with at the Multiorgan Transplant Program at the University of Western Ontario (UWO) have been assessing novel strategies for donor tissue and cell modification in transplantation, as this represents a complementary approach to T cell mediated tolerance in promoting graft survival. The theme of my proposed research will be to further elucidate the protective roles of vascular derived mediators, specifically the newly discovered biologically active molecule hydrogen sulphide (H_2S), in kidney transplantation and to determine the mechanisms by which H_2S may confer cellular protection and prolong renal allograft survival. In brief, the overall objectives of my research are as follows:

Years 1 and 2: a) Develop a model to evaluate the effects of H_2S on cold renal ischemia/reperfusion injury with respect to cellular injury, apoptosis, and inflammation through modifications in renal preservation solution and increased lengths of the cold ischemia time; b) Investigate the underlying mechanisms of our findings through *in vitro* studies on isolated renal vasculature.

Years 2 and 3: Explore hydrogen sulphide's contributions to graft inflammation and vascular dysfunction during acute T-cell mediated rejection and establish its role in the development of chronic allograft nephropathy through the use of a novel pre-sensitized "memory" mouse model to track alloreactive $CD4^+$ memory T cells.

Recent Relevant Research by Applicant:

The applicability of the role of H_2S in ischemia/reperfusion injury (IRI) and inflammation with respect to transplantation resides in its ability to confer protection from tissue ischemia and cellular apoptosis in both *in vitro* and *in vivo* models in other organ systems as well as in hibernation. These attributes, combined with its known interactions with previously described modulators of IRI such as nitric oxide, as detailed below, provide compelling evidence to suggest H_2S as another candidate in promoting allograft survival in the peri-transplant period and as a potential player in delaying the onset of chronic allograft nephropathy. The experience I have acquired throughout my PhD elucidating the effects of vascular derived mediators on renal hemodynamics and function and my clinical research in transplant ischemia reperfusion injury, combined with my post-doctoral training in the field of $CD4^+$ T cells in modulating immune tolerance will enable me to achieve these objectives.

My doctoral training has given me the unique opportunity to make a significant contribution to establishing the importance of nitric oxide (NO) in modulating cardiovascular and renal physiology throughout maturation(1-6). During these experiments we showed that the reactive renal hyper-perfusion which occurs following warm renal ischemia due to high-dose intravenous phenylephrine administration is diminished by the inhibition of NO synthesis, and that this phenomenon appears to be age-dependent. This data provided one of the earliest accounts that NO may be involved in IRI. In keeping with my interest in IRI, I worked with Dr. Patrick Luke at UWO to recently show that verapamil, a calcium channel blocker used to treat hypertension, supplemented

into the cold renal perfusate significantly improved long-term but not immediate renal allograft function in transplanted cadaveric human kidneys(7). Our findings suggest that verapamil may be protective against IRI by curbing the early cascade of lipid peroxidation and free radical injury, especially in the period immediately following reperfusion(8). Collaborating with the ongoing research on IRI at the Multiorgan Transplant Program and UWO, which is unique in regards to the collective expertise in the field, is invaluable for the successful completion of my research objectives (9)(10)(11,12).

Additionally, my post-doctoral training in the laboratory of Dr. Donna Farber at the University of Maryland has been exceptional in that it has afforded me a unique set of knowledge and skills in experimental transplant immunology which are integral to the development and successful completion of the proposed set of experiments described below. Dr. Farber's research has been instrumental in developing our understanding of the role naïve and memory $CD4^+$ T cell subsets play in modulating long-term graft survival(13-16). Most recently, she developed a novel mouse model for generating polyclonal alloantigen-specific memory $CD4^+$ T cells through the adoptive transfer of primed alloreactive T cells into host mice. These "memory mice" serve as a clinically relevant system for pre-sensitized transplant patients and permit the ability to track the actions of alloreactive memory $CD4^+$ T cells in the recipient host(17); this model will form the cornerstone of my research objectives outlined under years two and three.

Background Information

PHYSIOLOGICAL ROLES OF HYDROGEN SULPHIDE

H_2S, a colorless gas with a half-life of only minutes in solution, has been implicated in numerous physiological processes ranging from hippocampal long-term potentiation and inflammation to blood pressure regulation and has recently gained acceptance into the small family of gaseous transmitters previously populated by NO and carbon monoxide(18,19)(20). Endogenous H_2S is primarily produced by the enzymatic degradation of L-cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE)(21-23) and is detectable in serum(24-26) and in urine(23). Enzymes responsible for H_2S production are tissue specific, such that the expression of CBS is 30 fold greater than CSE in the brain(27,28), whereas CSE appears to be the key enzyme in H_2S production in the periphery with highest concentrations seen in pulmonary and aortic vascular smooth muscle(29). Experiments in rats have clearly demonstrated that i.v. administration of soluble H_2S, through its effects on K_{ATP} and voltage gated calcium channels, NO and endothelial hyperpolarizing factor, has a negative cardiac inotropic effect(30) and leads to a significant decrease in mean arterial pressure (Appendix A: Figure 1) (29)(31)(32,33). The ubiquitous expression of CBS and CSE in the kidney, liver, brain, peripheral lymphocytes and vasculature convincingly implicate H_2S as a potential player in a variety of physiological and pathophysiological states. Its role in the peri-transplant period has not yet been determined.

THE ROLE OF GASEOUS TRANSMITTERS IN RENAL TRANSPLANTATION

The period around the time of renal transplantation is associated with significant ischemia to allograft renal tubular epithelial cells, to endothelial cells (EC) and to vascular smooth muscle cells (VSMC) which lead to severe inflammation and eventual apoptosis once reperfusion is ensued. Experimental evidence suggests that oxidative stresses caused by ischemia/reperfusion injury (IRI) result in an increased release of cytokines, chemokines and growth factors which lead to delayed graft function, acute tubular necrosis, and chronic renal cellular remodelling which ultimately progress to early graft loss(34). In recent years, the involvement of NO in contributing to the apoptotic effects seen in IRI has come to light, as reported by Jevnikar *et al* at UWO(35).

Elucidating the mechanisms behind IRI and establishing novel methods to diminish the short and long term cellular injury sustained from it during the peri-transplant period is of paramount concern to preserving long-term graft function. Although numerous studies highlight the contribution of other vascular derived mediators including NO, endothelin-1 and CO releasing molecules to this cause, the potential influence of the newly described mediator, H_2S on renal IRI is limited yet promising(36,37). A recent study in a warm renal IRI model demonstrated that the application of topical H_2S results in decreased acute tubular necrosis (ATN), and limits the activation of protein markers of apoptosis and inflammation. Importantly, these authors also showed that although H_2S does not appear to influence renal function under baseline conditions, its protective role on renal function is significantly magnified in the face of IRI(36). In confirmation, additional reports in cardiovascular and neurological systems as well as in hibernation literature have also demonstrate exogenous and endogenous hydrogen sulphide's ability to limit the cellular damage and vascular remodelling that occur secondary to hypoxia and free radical induced EC and VSMC dysfunction(38-42). Given that H_2S is produced primarily from VSMC, it is not surprising that the decrease in CSE following VSMC injury results in an imbalance between protective and destructive effects of local mediators. Taken together, these data provide compelling evidence to suggest that H_2S, either alone or in conjunction with other vascular derived mediators such as NO, may be protective against the cellular changes and remodelling observed following renal IRI in the peri-transplant period. My previous experience in NO physiology and IRI will be invaluable in evaluating this theory.

ROLE OF H_2S IN INFLAMMATION

Chronic allograft nephropathy (CAN) is the result of immunological and non-immunological injury, including acute rejection episodes, hypoperfusion, IRI, calcineurin toxicity, infection and recurrent disease. Although the development of CAN is often insidious, it may be preceded by subclinical rejection in a well-functioning allograft. The hallmark histological findings of CAN include interstitial fibrosis, tubular atrophy, glomerulosclerosis, fibrointimal hyperplasia and arteriolar hyalinosis (43).

Although the mechanisms behind CAN are complex, the additive effects of IRI sustained in the early peri-transplant period, compounded with episodes of graft inflammation and rejection may be of significance to long-term graft survival. Apart from the convincing evidence that suggests a protective role for H_2S against EC dysfunction and VSMC proliferation and hyperplasia seen after damage induced by reactive oxygen species (superoxide anions, H_2O_2 , $ONOO^-$ and hydroxyl radicals) observed in IRI (37-39,44), its role in tissue inflammation has recently been described. Leukocyte adherence and migration are the earliest events in the inflammatory process, as well as in acute allograft rejection(45). Recent experiments in rodent models of mesenteric inflammation and gastropathy have reported that H_2S inhibition results in increased expression of the adhesion molecule ICAM-1 which is known to increase leukocyte adherence (46,47) and $CD4^+$ T lymphocyte activation(48). The link between $CD4^+$ T cell mediated injury in triggering vascular remodelling is strengthened by reports showing the prominence of these lymphocytes in atherosclerotic plaques(49,50) likely due to EC and VSMC dysfunction by iNOS and TGF-β over-expression(51,52). The eventual stenosis of graft conduit arteries leading to ischemia and parenchymal fibrosis seen in CAN is commensurate with the pathophysiology of atherosclerotic plaques. Importantly, the number and severity of acute rejection episodes as well as the persistence of resident renal interstitial T lymphocytes following acute rejection correlates with a decreased probability of long-term renal allograft survival and CAN(53,54), thus supporting the potential importance of $CD4^+$ T cells in modulating the destruction of renal VSMC over time and eventually leading to CAN(13,55). Although the place of H_2S in these events is not yet determined,

experimental evidence suggests a dose-dependent inhibitory effect of H_2S on antigen induced $CD4^+$ T cell proliferation and IL-2 production(56), both of which are key steps in acute cellular rejection. Amalgamation of evidence from various disciplines suggests that vascular changes of CAN may be attributable to a gradual decline in the baseline production in local H_2S from VSMC due to early IRI and T lymphocyte mediated apoptosis following episodes of acute rejection through the effects of iNOS and TGF-β upregulation; this theory warrants further investigation.

Hypotheses, Research Methods and Analysis

YEAR 1 AND 2: DETERMINING THE ROLE OF H_2S IN ABROGATING THE EFFECTS OF ISCHEMIA-REPERFUSION INJURY

Although most of our knowledge in renal IRI is based upon warm ischemic protocols, clinical transplantation utilizes cold ischemia to limit cellular injury. However, evidence suggests that grafts with cold ischemia times of more than six hours develop more severe vascular endothelial injury, tubular necrosis and VSMC dysfunction compared with grafts with shorter preservation times(57). Based upon previous reports which highlight the cellular protective role of H_2S, especially in the warm renal IRI model, the objective of the first series of experiments will be to determine the effect of H_2S in a novel murine IRI model consistent with circumstances observed in clinical renal transplantation. **It is hypothesized that H_2S plays a significant role in limiting IRI following transplantation of kidneys placed in cold renal preservation solution. Additionally, it is predicted that as cold ischemic time lengthens, the presence of H_2S will become more important in protecting against cellular injury.**

a) Assessing the effect of supplemental H_2S to cold renal preservation solution: These hypotheses will be tested using the transplantation of syngeneic donor kidneys (in order to eliminate any confounding effects of immunosuppression) into Balb/c mice who have received bilateral nephrectomies (Appendix A: Figure 2). Donor kidneys will either be flushed with cold University of Wisconsin (UW) preservation solution or UW + the soluble H_2S releasing molecule GYY4137 (58) at the time of procurement as well as during cold storage. Kidneys will be cold stored for various lengths of time prior to transplantation (4h, 8h, 12h, 16h, 20h, 24h, 36h and 48h) to accurately mimic extended cold ischemic times observed in actual clinical settings. After reperfusion, intravital microscopy will be utilized to assess renal cortical and medullary blood flow and inflammatory cell trafficking. We will monitor urine and blood at several time points to assess various parameters of renal function and tissue hypoxic injury. At the time of sacrifice, allografts will be histologically scored to assess degree of ATN, glomerular and vascular injury. Homogenates will be assessed for protein markers for inflammation (NF-κB, iNOS, ICAM-1, TGF-β), apoptosis (caspace-3, Bid, Bcl-2, JNK1/2 and ERK1/2), H_2S production (CSE) and VSMC quantification (SM22-α, α-smooth muscle actin and smooth muscle myosin heavy chain).

b) Investigating the underlying protective mechanisms of supplementing cold preservation solution with H_2S through *in vitro* studies on isolated renal vasculature: Data from the above experiments will then be used to develop an *in vitro* protocol to further characterize the effect of pre-treatment with supplemental H_2S on isolated, perfused renal arteries obtained from murine kidneys exposed to select lengths of cold ischemic times. Isolated vessels will be tested with various pharmacological perturbations including contractility studies using phenylephrine and NOS blockade. Histological, morphometric and immunohistochemical analyses will also be carried out (similar to experiment described above) to assess intimal and medial cross-sectional areas, CSE levels as well as estimates of cellular proliferation using BrdU incorporation and apoptosis using

TUNEL assay. Similar series of *in* vitro analyses will be carried out on isolated renal vasculature at 7 days following transplantation of kidneys exposed to either H_2S supplemented or control cold renal perfusate.

YEAR 2-3: ESTABLISHING THE INFLUENCE OF H_2S IN CHRONIC ALLOGRAFT NEPHROPATHY

The second and third years of my research plan will be spent exploring hydrogen sulphide's contributions to graft inflammation during acute T-cell mediated rejection and establish its role in the development of CAN. To achieve these aims, experiments will be carried out to test the **hypothesis that a chronic decrease in H_2S production mediated by the destruction of VSMC by the infiltration of memory $CD4^+$ T cells during acute rejection is responsible for the local fibrosis and vascular remodelling and eventually leads to chronic allograft nephropathy.**

The experimental protocol for these experiments can be seen in Appendix A: Figure 3. Briefly, six groups of "mosaic memory Balb/c mice" will be created through the generation of polyclonal alloantigen-specific memory CD4 T cells which are then adoptively transferred into hosts. After undergoing bilateral native nephrectomy, mice will receive kidneys obtained from mismatched C57BL/6 mice and placed under an immunosuppressive protocol as described in the figure using cyclosporine A (CyA) with and without the H_2S releasing molecule GYY4137. The group receiving inadequate immunosuppression to actively stimulate acute rejection will have their episode of rejection treated and then immunosuppression will be withdrawn. This particular group of mice will then be allowed to reject their allograft completely allowing for the development of histological changes comparable to those seen in CAN. Data will be collected on a daily basis on intake and output. At 120 days, animals will be sacrificed for histological, Western Blot and immunoflurorescence analysis. In addition, renal vascular and interstitial homogenates will be assessed for both naïve and memory $CD4^+$ T cell phenotype and function using FACS analysis and cytokine quantification, respectively. In addition, we will utilize laser capture microdissection, which allows for the detailed study of circumscribed cell groups, to isolate endothelial, renal tubular epithelial and vascular smooth muscle cells from fresh samples obtained from the renal cortex and medulla in the above kidneys. Protein expression of markers for apoptosis, inflammation, H_2S production and tissue remodelling will be assessed in these cell types, as previously listed above.

Summary and Future Directions

Apart from first defining the role of H_2S in renal IRI following cold organ preservation as well as in chronic allograft nephropathy, these series of experiments will pave the way for further experimental and translational studies as our findings will be equally applicable to other organ systems including heart, lung, liver, small bowel and pancreas which are all more susceptible to ischemic injury. Concomitant translational studies will include the use of the LifePort® machine (a donor kidney pulsatile-perfusion and cold storage transport device) to assess the applicability of our experimental findings in a clinical transplant setting. Data from the first two years of my research will be used to apply for funding and operational support through the Kidney Foundation of Canada and CIHR. The pursuit of an oral, long-acting form of H_2S donor is currently underway. If these experimental and early clinical results are successful, I am very interested in developing a randomized, multicenter, controlled trial to assess whether this oral supplement, in addition to ongoing immunosuppression, may prove to be a revolutionary method of mitigating the deleterious effects of IRI and CAN, thus prolonging allograft survival.

APPENDIX A

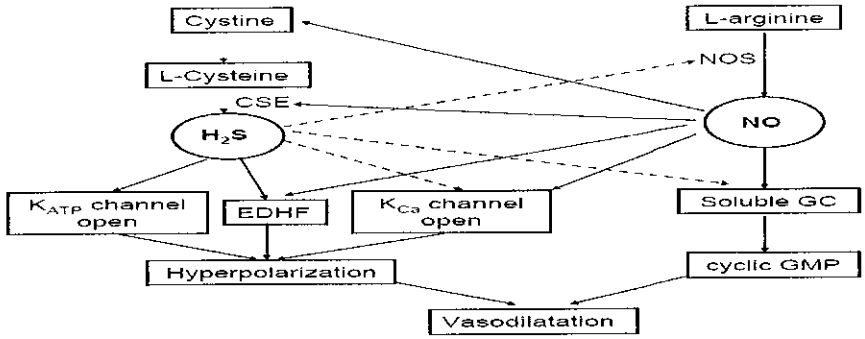

Figure 1. Potential interactions between H_2S and NO in mediating vascular tone. Solid arrows delineate a positive influence whereas dotted arrows indicate a negative effect.
Adapted from Wang 2002(20).

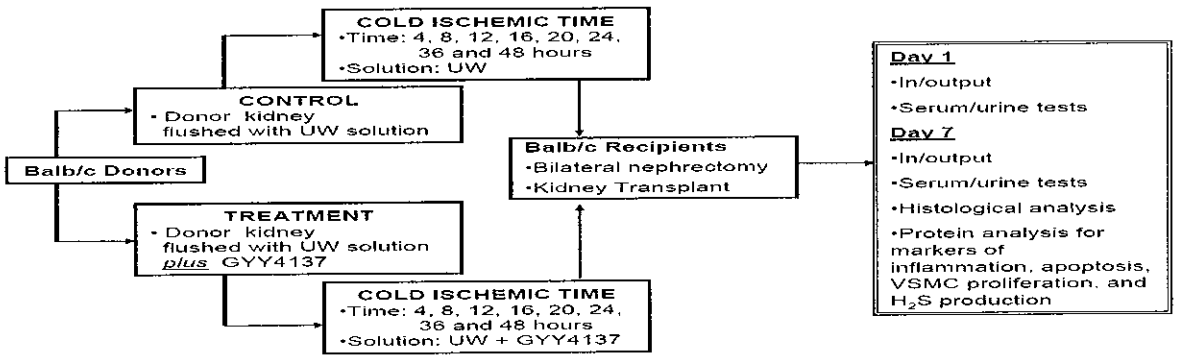

Figure 2. Assessing the effect of supplemental H_2S to cold renal preservation solution

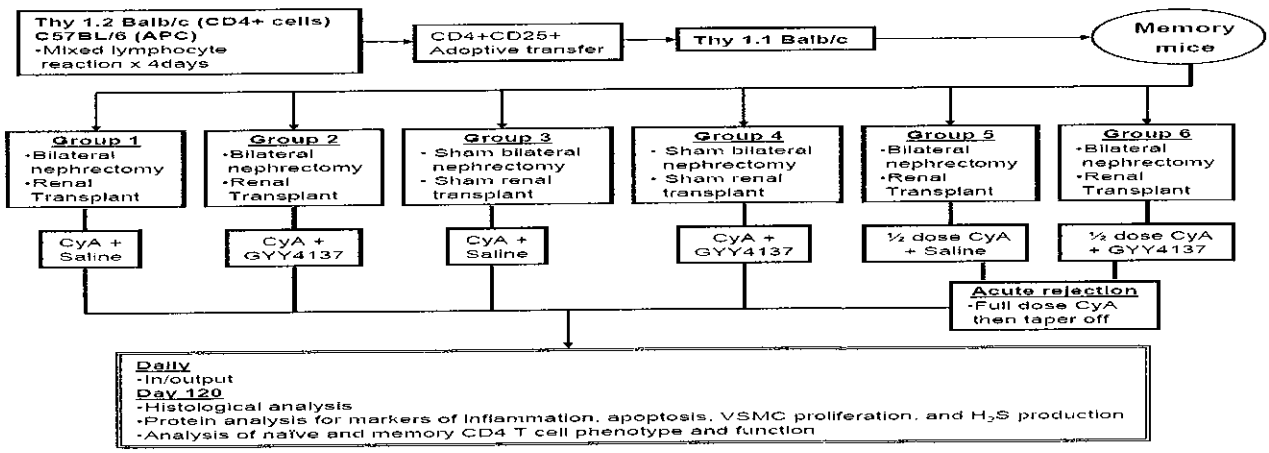

Figure 3. Determining the effect of supplemental H_2S in acute and chronic rejection

Anti-Thymocyte Globulin Project

At the time of renal transplantation, patients receive a variety of immunosuppressive medications to minimize the potential for acute rejection episodes in the newly transplanted kidney. Transplant immunosuppressive medications are classified into two broad categories which are induction and maintenance therapy. Induction is achieved through the use of extremely potent medications that act very fast to suppress the immune system by acting on a particular type of white blood cell line called T-cells. This initial medical therapy is then followed by maintenance therapy, where a different set of medications are used to keep the recipient's defenses from attacking and eventually rejecting the donor organ.

One of the mainstays of induction immunotherapy in the early time period around transplantation is the use of agents which target circulating T-cells including anti-thymocyte globulin (Thymoglobulin) and basiliximab (Simulect). Although both these agents are widely utilized across all transplant centers to effectively deplete and inhibit T-cell populations, respectively, their effects on how the T-cell populations recover from the initial suppression are not well understood. Experimental evidence from animal studies suggests that various sub-populations of T-cells may be more sensitive to induction therapy than others and that their rapid recovery/repopulation may lead to imbalances in the proper functioning of the immune system. This novel finding may be a potential explanation for the elevated rates of acute rejection observed in highly sensitized patients (ie: patients who have had multiple transplants or numerous blood transfusions) despite adequate immunosuppression and may highlight the importance of monitoring patients to ensure that they do not develop disorders which stem from an overactive immune system including diabetes and graft versus host disease.

The purpose of the current study is to assess T-cell reconstitution characteristics and functional properties over time in patients who receive Thymoglobulin or Simulect induction therapy and determine whether a similar pattern of T-cell recovery is observed as in animal studies. Findings from these studies will be extremely important in developing new immunosuppressive strategies to promote both short-term and long-term kidney graft function in kidney transplant recipients. In addition, the results obtained from this study may help future transplant professionals in predicting the occurrence of new-onset immune disorders in the period around the time of transplantation.

Hydrogen Sulphide Project

The nature of transplantation leads to tissue injury as organs are damaged by the loss of blood supply and ischemia associated with the procurement procedure. The potential benefit of donor tissue and storage modification to protect organs has not been intensively investigated as mainstream approaches to improving transplant survival remains focused on pharmacological inhibition of immune cell activation. The theme of my research is to further elucidate the protective roles of vascular derived mediators, specifically the newly discovered biologically active molecule hydrogen sulphide (H_2S), in kidney transplantation and to determine the mechanisms by which H_2S may confer cellular protection and prolong renal allograft survival. In brief, the overall objectives of the experiments ongoing in our laboratory are to develop a model to evaluate the effects of H_2S on cold renal ischemia/reperfusion injury with respect to cellular injury, apoptosis, and inflammation through modifications in renal preservation solution and increased lengths of the cold ischemia time as well as to investigate the underlying mechanisms

of our *in vivo* findings through *in vitro* studies on human umbilical vein endothelial cells (HUVEC) and renal tubular epithelial cells (TEC). With respect to the *in vitro* experiments, we will evaluate the effect of pre-treating HUVEC and TEC with either PBS or PBS + the H_2S donor GYY4137 or NaHS at varying concentrations prior to placing them in a cold (4°C) hypoxia chamber for 15 or 30 minutes. Following re-oxygenation for 24h, the cells will be stained with annexin-V and 7-AAD to determine degree of cellular apoptosis and necrosis, respectively and analyzed using flow cytometry. Western blot analysis will be used to quantify iNOS, CSE, Platelet activating factor, TGF- β and ICAM-1 protein expression.

Apart from establishing the first description of the role of H_2S in renal IRI following both cold and warm organ preservation, these series of experiments will pave the way for further experimental and translational transplantation studies in other organ systems including pancreas and heart. If the current studies are successful, further investigations into the role of H_2S in modulating long-term renal allograft function and hence minimizing chronic allograft nephropathy are also planned using newly emerging orally bioavailable H_2S donor molecules.

Cell Biology

ATCC® Number:	**CRL-1730™**	Order this Item	Price:	**$280.00**

Designations:	HUV-EC-C
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	endothelial
Source:	**Organ:** umbilical vein **Tissue:** vascular endothelium **Disease:** normal **Cell Type:** endothelial
Cellular Products:	factor VIII [23284]
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (technology from amaxa)
Tumorigenic:	No
DNA Profile (STR):	Amelogenin: X CSF1PO: 11,12 D13S317: 9,11 D16S539: 11,12 D5S818: 11,12 D7S820: 8,12 THO1: 6,9.3 TPOX: 8,11 vWA: 16
Cytogenetic Analysis:	This is a hypodiploid human cell line. The modal chromosome number was 45 occurring in 72% of cells counted. The rate of polyploid cells was 15.8%. All cells had monosomic N13 and the subclone with additional monosomic N15 predominates. Other coexisting subclones include those with 46,XX,-11,-13,i(11p),i(11q) and 46,XX,+11,-13 karyotypes. Both X chromosomes appear normal.
Comments:	Endothelial Cell Growth Supplement (ECGS) and unidentified factors from bovine pituitary, hypothalamus or whole brain extracts are mitogenic for this line. [23284] The cells have a life expectancy of 50 to 60 population doublings.

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PT 2 cells

PT cells isolation from urine and basic culture

The procedure for isolation of a specific culture of proximal tubule epithelial cells from urine samples was modified from methods described by Inoue et al. (2001)[65] and Laube et al. (2005)[66]. Urine samples for this procedure were obtained from the principal investigator and collected at various times of the day in volumes ranging from 24 hours collections (~1.5 L) to individual voiding (~100 ml). In addition, anonymous urine samples were collected from in-house patients under the care of Drs. John Crocker or Phillip Acott. Anonymous patient samples were collected with ethical approval for the use of discarded or waste material (no signed patient consent was required). The urine samples were collected by an attending nurse and delivered unlabeled to the Division of Nephrology research laboratory for immediate use. Anonymous urine samples were typically 75-100 ml in volume.

Upon receiving the sample, 15 ml portions were poured into two 15-ml centrifuge tubes and centrifuged at 300g for 5 minutes. Following centrifugation the supernatant was discarded and fresh aliquots were added to each tube. This process was repeated in the same two centrifuge tubes until the entire urine sample was used. The cell pellet was washed once with DMEM supplemented with 10% FBS and 100 IU/ml penicillin/ 100 µg/ml streptomycin, resuspended, and recentrifuged at 300g for 5 minutes. The

supernatant was removed by aspiration and the two cell pellets were resuspended in growth media and pooled in a single tube. The cell suspension was plated in 3.5 cm dishes (Falcon) coated with collagen type-I (see section 2. 2. 2.).

The growth media for the first 24 hours consisted of DMEM supplemented with 20% FBS and 100 IU/ml penicillin/ 100 µg/ml streptomycin. Cells were incubated overnight at 37°C in a humidified 95% air/5% CO_2 environment. The following day the media was aspirated and replaced with fresh DMEM supplemented with 10% FBS and 100 IU/ml penicillin/ 100 µg/ml streptomycin. Cells were cultured until approximately 80% confluent before being trypsinized (see section 2. 2. 1.), resuspended in growth media, and plated in 50-ml collagen coated flask for further growth. The cells were subdivided by trypsinization as necessary.

PT Cell Isolation and Culture

Centrifugation of urine samples from a healthy adult (i.e. the principal investigator), collected in various volumes and at various times of the day, did not yield any viable cells. The majority of the samples did not provide a visible cell pellet. Pelleted material that was obtained consisted mostly of debris when resuspended in growth media and examined microscopically.

Isolation of cells from fresh urine samples collected anonymously from ill individuals on the IWK ward did provide a stable cell culture. Of the several urine samples that were examined one sample did provide a visible cell pellet and viable growth. Morphologically these cells appeared uniformly elongated and spindle-shaped. Unfortunately an image of these cells is not available to illustrate their morphology.

Initial growth of these cells, designated PT1, was restricted to small island-like groups of cells that eventually grew to merge with one another. The PT1 cells did not divide rapidly; they had a doubling time of approximately 3-4 days, with a decreased rate of division as the culture neared confluency. When confluency was reached the culture maintained a monolayer arrangement; no piling of cells was observed. The PT1 cells were put through seven passages before active division ceased and the culture senesced.

When the PT1 cells had survived several passages a single flask was prepared in K-SFM (Fig. 17) rather than 5 or 10% DMEM. These cells took on a different morphology and much decreased growth rate from what was observed when cultured in DMEM. The cells lost their elongated spindle shape in favour of a more spherical morphology with the majority of cells displaying spindle-like projections similar to those observed with the HK-2 cells. The cells were able to grow to confluency with a doubling time of approximately 7-8 days, as long as media changes were performed every two days over this period. The less frequently the growth media was changed the slower the cells appeared to divide. PT1 cells were maintained in K-SFM for several passages throughout which the same morphology and growth rate was observed.

After the PT1 cells were put through a single passage in K-SFM a flask of cell were returned to culturing in 5 or 10% FBS DMEM. Returning the PT1 cells to DMEM did not restore the morphology and growth characteristic originally observed. Due to the change in characteristics and behaviour these cells were designated PT2 (Fig. 18). While the original PT1 cells underwent senescence after several passages, the PT2 cells underwent numerous additional passages without indication of reduction in activity. The growth of the PT2 cells was much more robust than the PT1 cells, with a doubling time of

approximately 1-2 days (in 5% FBS DMEM). The PT2 cells easily grew to confluency as a monolayer, but if neglected would pile upon each other. The morphology of the PT2 cells (Fig. 19) were more elongated than the HK-2 cells, but not uniformly so as observed with the PT1 cells. A fraction of PT2 cells were also spherical in shape with spindle-like projections similar to the HK-2 cells.