Injury and Intestinal Barrier Dysfunction: Past, Present, and Future

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Background

- The intestine plays a significant role in the systemic inflammatory response (SIRS)
- SIRS can lead to distant organ injury, multi-organ failure, and death
- Our understanding of the gut’s role in causing SIRS has evolved over the past several decades
Bacterial Translocation

- 1980’s: Gut Origin of Sepsis
  - Passage of luminal bacteria (endotoxin) into portal circulation\(^1\)
  - Bacteria found in mesenteric lymph nodes\(^2\)
  - Bacteria reaches systemic circulation via portal vein
    - Kupffer cells produce cytokines
  - Systemic Inflammatory Response

Bacterial Translocation

• Early 1990’s: Bacterial Translocation in question

• Moore, et al: Is there enteric bacteria in the portal blood of severely injured trauma patients?
  – 20 injured patients requiring emergent laparotomy
  – Portal vein catheters inserted
  – Blood drawn up to 5 days post-operatively
  – 8/212 (2%) of blood cultures positive
    • 7 presumed contaminants
    • 1 S. Aureus in patient with known S. Aureus pneumonia
  – Conclusion: No portal or systemic bacteremia despite 30% incidence of MOF in these patients

"We look hard at what is routinely done in the Shock Trauma ICU and ask, 'How does this treatment affect the gut function?' We are finding that when a person is critically ill, the gastrointestinal (GI) tract doesn't work. If we can make the gut work better, then we can prevent a lot of infection,"

Gut Inflammation

- 1990’s-Present: Gut Inflammation
  - Gut barrier breakdown causes intestinal inflammatory response
    - Intestinal cytokine production
  - Gut-derived inflammatory mediators carried in intestinal lymph
  - Activated intestinal lymph causes SIRS, distant organ injury

1. Deitch, EA. *Surgery.* 2002;131:241-244
TRAUMA–SHOCK

- Decreased intestinal blood flow and altered intestinal permeability
- Bacterial translocation
- Systemic infection

Increased portal endotoxemia

Systemic Endotoxemia

Septic state

Distant organ failure (MODS)

SHOCK–TRAUMA–INDUCED DECREASE IN GUT BLOOD FLOW

- Gut ischemia–reperfusion injury
- Loss gut barrier function (Bacterial translocation)

Gut–derived inflammatory factors carried in the mesenteric lymph

Gut inflammatory response

Septic state

Distant organ failure (MODS)
Mesenteric lymph from burned animals:
  - Activate PMNs
  - Activate endothelial cells

Portal vein plasma did not activate PMNs

Intravenous Injection of Trauma-Hemorrhagic Shock Mesenteric Lymph Causes Lung Injury That Is Dependent Upon Activation of the Inducible Nitric Oxide Synthase Pathway

Maheswari Senthil, MD, Anthony Watkins, MD, Dimitrios Barkos, MD, Da-Zhong Xu, MD, PhD, Qi Lu, MD, Billy Abunganu, BSc, Frank Caputo, MD, Rena Feinman, PhD, and Edwin A. Deitch, MD

• Lymphatic Duct Ligation (LDL)
  – Decreases histologic lung injury
  – Decreases lung permeability
  – Decreases neutrophil CD11b expression

• Mesenteric lymph flow depends on depth of shock

• Maximal PMN priming by mesenteric lymph occurs in the 3rd hour post-shock

• Activity of mesenteric lymph depends on depth and duration of shock

Arachidonic acid in postshock mesenteric lymph induces pulmonary synthesis of leukotriene B₄

Janeen R. Jordan,¹,² Ernest E. Moore,¹,² Eric L. Sarin,¹,² Sagar S. Damle,¹,² Sara B. Kashuk,³ Christopher C. Silliman,¹,² and Anirban Banerjee³

[Bar charts showing comparisons between pre- and postshock lymph flow rates and effects of PSML and PSML + LTB4 Receptor Antagonist.]
RINGER’S LACTATE

- Current standard resuscitation regimen
  - Potentiates neutrophil activation
    - Rhee et al. 1998
  - Contributes to end organ injury
    - Savage et al. 2005
Pentoxifylline (PTX)

- Non-specific Phosphodiesterase Inhibitor
  - Increases cyclic AMP
  - PKA activation

- Clinical Applications:
  - Intermittent Claudication
  - Alcoholic Hepatitis

- Animal Models:
  - Decreases pro-inflammatory cytokine activation
  - Attenuates neutrophil oxidative burst
  - Decreases distant organ injury
Hemorrhagic shock

Lactate

TIME (min)

0 60 120

mmol/L

HTS LR LR + PTX

Bacterial Translocation

% 80

HTS LR LR+ PTX

Hemorrhagic Shock

Classic treatment

Ringer’s Lactate

Proposed treatment

Hypertonic Saline + Pentoxifylline

- Improves microcirculation
- Attenuates oxidative stress
- Downregulates neutrophil function
- Reduces host organ injury
Hemorrhagic Shock

Ringer’s Lactate (RL)
- Potentiate neutrophil activation
  *Resuscitation 2004*
- Promote endothelial dysfunction
  *J Trauma 2005*
- Contribute to end organ injury
  *J Trauma 2006*

HSPTX
- Reduce oxidative stress
- Downregulate PMN function
  *J Trauma 2005*
- Attenuate Post-shock Lung Injury
  *J Trauma 2006*
HSPTX Protects Against Hemorrhagic Shock Resuscitation-Induced Tissue Injury: An Attractive Alternative to Ringer’s Lactate

Ronal Coimbra, MD, PhD, FACS, Rafael Porcides, MD, William Loomis, BS, Heidi Melbostad, BS, Rohan Lall, MD, Jessica Deree, MD, Paul Wolf, MD, and David B. Hoyt, MD, FACS

Sham

Ringers Lactate

HSPTX

Nitric oxide and Ischemia Reperfusion

• iNOS induction and production of sustained quantities of NO occur in the gut after I/R injury.

• Nitric oxide
  
  Direct effects on cell signaling: Transcription factor activation (NF-κB and STAT3) and cytokine production (TNF-α and IL-6)

  *J Exp Med 1998*

  Indirect cytotoxic effects: Peroxynitrite formation
Intestinal I/R Injury

iNOS

NO

NF-κB/STAT3

Peroxynitrite

TNF-α, CINC, IL-6

Organ Injury
Hypothesis

- The attenuation in gut injury observed with HSPTX after hemorrhagic shock is associated with a decrease in intestinal iNOS activity and NO-mediated events including local pro-inflammatory cytokine production when compared to RL in vivo.
Methods

- **RL**: 32 mL/kg racemic RL (n=7)
- **HSPTX**: 4 mL/kg 7.5% NaCl + PTX 25 mg/kg (n=7)
- Sham group (n=5)
iNOS Content

Ileal iNOS Content (Pixel Total + SEM)

Sham | RL | HSPTX

0 | 20000 | 40000 | 60000 | 80000 | 100000 | 120000

* P < 0.05

130 kD

* P < 0.05
Nitrite Concentration (µmol/L + SEM)

- **Sham**: Lower concentration
- **RL**: Higher concentration
- **HSPTX**: Moderate concentration

* * P < 0.05
Cytoplasmic I-κBα Phosphorylation

**Sham RL HSPTX**

I-κBα Phosphorylation (Pixel Total + SEM)

- **Sham**: Low phosphorylation level
- **RL**: High phosphorylation level
- **HSPTX**: Moderate phosphorylation level

*P < 0.01

41 kD
Nuclear NF-κB Phosphorylation

NF-κB p65 Phosphorylation (Pixel Total + SEM)

Sham  RL  HSPTX

65 kD

* P < 0.01
STAT-3

Graph showing ileal STAT3 phosphorylation (Pixel Total + SEM) for Sham, RL, and RL+PTX conditions. The RL condition shows significantly higher phosphorylation compared to Sham and RL+PTX. Immunoblot images confirm these findings for P-STAT3 and STAT3.
TNF-α Concentration (pg/mL + SEM)

- Sham
- RL
- HSPTX

* P < 0.01
Interleukin-6

IL-6 Concentration (pg/mL + SEM)

Sham | RL | HSPTX

* P < 0.01
Intestinal I/R Injury

iNOS

HSPTX

NO

HSPTX

NF-κB

HSPTX

TNF-α, CINC, IL-6

HSPTX

Peroxynitrite

Organ Injury
Hypertonic Saline and Pentoxifylline Attenuates Gut Injury After Hemorrhagic Shock: The Kinder, Gentler Resuscitation

Jessica Dere, MD, Tercio de Campos, MD, Edna Shenvi, BS, William H. Loomis, BS, David B. Hoyt, MD, and Raul Coimbra, MD, PhD

Phosphodiesterase inhibition downregulates intestinal injury and inducible nitric oxide synthase activity after hemorrhagic shock

JESSICA DERE, WILLIAM H. LOOMIS, JAMES G. PUTNAM, PAUL WOLF, TODD COSTANTINI, DAVID B. HOYT and RAUL COIMBRA

TNF-α and Intestinal Barrier

The pivotal role of tumor necrosis factor-alpha in signaling apoptosis in intestinal epithelial cells under shock conditions.

Diebel LN, Liberati DM, Baylor AE 3rd, Brown WJ, Diglio CA

J Trauma. 2005 May;58(5):995-100

L. Diebel, MD
Gut Barrier Breakdown

• 2008: Intestinal Barrier Injury
  – Can we prevent the intestinal inflammatory response and subsequent SIRS by limiting intestinal barrier breakdown?

• Intestinal Tight Junction
  – Creates physical barrier that seals the space between adjacent epithelial cells
  – Regulates intestinal permeability
  – Modulation of tight junction proteins alters epithelial barrier function

Turner JR. Am J Pathol. 2006;169:1901-1909
Normal Intestinal Barrier

Table. Protective mechanisms of the intestine

<table>
<thead>
<tr>
<th>Mechanical</th>
<th>Nonmechanical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peristalsis</td>
<td>Normal gut flora–mediated colonization resistance</td>
</tr>
<tr>
<td>Epithelial barrier</td>
<td>Secretory immunoglobulins</td>
</tr>
<tr>
<td>Mucus layer</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>Tight junctions</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td></td>
<td>Antigen receptors</td>
</tr>
</tbody>
</table>

Intestinal Tight Junction

- **Occludin**
  - Four transmembrane domains
  - Attaches adjacent cells at tight junction

- **ZO-1**
  - Attaches occludin to perijunctional actin cytoskeleton

- **Myosin light chain kinase (MLCK)**
  - Increases phosphorylation of myosin light chain (MLC)
  - Modulates contraction of the actin cytoskeleton
Caco-2 cells + Cytomix

Costantini T, et al., *Life Sciences*, 2009
Caco-2 cells + Cytomix

Costantini T, et al., *Life Sciences*, 2009
Phosphodiesterase inhibition attenuates alterations to the tight junction proteins occludin and ZO-1 in immunostimulated Caco-2 intestinal monolayers


Life Sciences 84 (2009) 18–22

Costantini T, et al., Life Sciences, 2009
Burn-induced Histologic Gut Injury

Intestinal Occludin

6 hour

\[ \text{Sham} \rightarrow \text{Burn} \rightarrow \text{Burn/PTX} \]

\[ \text{Relative Band Density (±SEM)} \]

24 hour

\[ \text{Sham} \rightarrow \text{Burn} \rightarrow \text{Burn/PTX} \]

\[ \text{Relative Band Density (±SEM)} \]

Occludin

\[ \beta\text{-actin} \]

* \( p < 0.01 \) vs. Sham
† \( p < 0.05 \) vs. Burn
‡ \( p < 0.01 \) vs. Sham
§ \( p < 0.05 \) vs. Burn

Intestinal ZO-1

6 hour

24 hour

Relative Band Density (+/- SEM)

Sham  |  Burn  |  Burn/PTX

ZO-1

β-actin

* p < 0.01 vs. Sham

† p < 0.05 vs. Sham

‡ p < 0.05 vs. Burn

Tight Junction Confocal Microscopy

Burn-induced intestinal permeability to 4kDa FITC-Dextran.

* $p < 0.001$ vs. Sham
† $p < 0.001$ vs. Burn

Intestinal Barrier Breakdown

- Myosin light chain kinase (MLCK)
  - Increases phosphorylation of myosin light chain
  - TNF-α increases MLCK expression
  - Increased MLCK protein expression:
    - Decreases ZO-1 and occludin levels
    - Increases intestinal permeability

Intestinal Barrier Breakdown

- Intestinal NF-κB
  - NF-κB mediates activation of MLCK by binding to MLCK promoter
  - Inhibition of NF-κB p65 decreases MLCK activation

Ye, et al. Am J Physiol Gastrointest Liver Physiol 2006;290:496-504
Methods

30% TBSA steam burn for 7 seconds

balb/c mice

IP injection:
12.5mg/kg PTX in 500 μl Normal Saline vs. 500 μl Normal Saline

2hr

4hr

Intestinal Permeability:
4 kDa FITC-Dextran

Harvest Distal Ileum:
Histology
TNF-α ELISA
Confocal Microscopy
- Phosphorylated MLC
Western blot
- MLCK
- Cytoplasmic IKK, IkBa
- Nuclear NF-κB p65
Intestinal Myosin Light Chain Kinase

* p < 0.02 vs. Burn
Cytoplasmic Phosphorylated IKK$\alpha$ / $\beta$

* p < 0.05 vs. Burn
Cytoplasmic Phosphorylated IkB α

* p < 0.01 vs. Burn
Nuclear NF-κBp65

* p < 0.03 vs. Burn

Relative Band Density (+/-SEM)

- **P-NF-κB p65**
- **Beta-Laminin**
Intestinal TNF-α

* p < 0.03 vs. Sham
† p < 0.05 vs. Burn
Phosphorylated MLC Confocal Microscopy

Costantini, et al.  *J Trauma* 2009

Bar = 20 µm
Intestinal Permeability 4 Hours Post-Burn

![Graph showing FITC-Dextran levels with Sham, Burn, and Burn/PTX groups.](image)
What is in the Future?
Future #1: Novel Imaging of Intestinal Injury

- Intraluminal placement of near-infrared dye
  - Alexa Fluor 680

- Imaging using Xenogen IVIS Lumina

- Quantification of fluorescence
  - Correlates with “classic” assays of intestinal injury and intestinal permeability
Near-infrared Imaging of Intestinal Injury

Sham 0hr Burn 4hr Burn 6hr Burn 24hr Burn 48hr Burn
Quantification of Near-infrared Imaging

Sham  4hr Burn

Abdominal Quantification

Intestinal Quantification
Gavage Time Course

<table>
<thead>
<tr>
<th>Min</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>4h Burn</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Gavage Quantification

Abdominal Quantification

Fluorescent Intensity (+/- SEM)

- Sham
- 4hr Burn

* p<0.03
Utilizing Phage Display Technology to Identify Peptide Sequences Targeting the Burn Injured Intestinal Barrier

Todd W. Costantini MD, Carrie Y. Peterson MD, James G. Putnam BS, Ritsuko Sawada PhD, William H. Loomis BS, Brian P. Eliceiri PhD, Andrew Baird PhD, Vishal Bansal MD, Raul Coimbra, MD, PhD
Background

- Intestinal injury is known to result from several clinical conditions resulting in significant morbidity and mortality
  - Severe trauma, burn
  - Inflammatory bowel disease
  - Necrotizing enterocolitis

- The ability to effectively target the intestinal mucosa to deliver biotherapies could be of powerful clinical utility
  - Prevent gut injury
  - Speed intestinal barrier healing
Drug Delivery

• Delivery of therapeutics to the intestinal mucosa remains a difficult problem

• Must be delivered to the cells of the intestinal wall in sufficient quantities to achieve the desired effect
  – Issues of clearance
  – Timing of drug delivery
  – Alterations in perfusion to the gut following injury
Phage Display

• Used to identify functional targeting ligands and their corresponding receptors.

• Diverse libraries of peptide sequences \(1 \times 10^{12}\) can be displayed by utilizing the bacteriophage M13.

• Single peptide sequence is displayed on a single phage
  – Allows for biopanning of a large number of peptide sequences

Phage Display

• Phage-based vectors can be used to identify peptides which can perform targeted delivery of biotherapeutics
  – Genes, antibiotics, growth factors

• Screen for peptides that home to specific tissues

• Wide-ranging applications
  – Cancer Therapies:
    • Targeting tumor vasculature with TNF-α \(^1\)
    • Screening for antigens overexpressed by carcinomas \(^2\)

Phage Display

M13 Phage

ssDNA

YPRLLLTP

M13 Phage

ssDNA

YPRLLLTP
Hypothesis

• We postulated that by utilizing in vivo phage display, we would identify peptide sequences which internalize into the intestinal epithelial following severe injury.

• We could bind this newly discovered peptide sequence to fluorescent nanoparticles in order to image its delivery into the gut barrier.
Methods- Phage Screening

30% TBSA steam burn for 7 seconds

balb/c mice

2hr

• Intestinal mucosa isolated 2 hours following burn
• Mucosa incubated with Phage library containing $10^{12}$ different peptide sequence
• Selected Phage amplified using E. coli
• Process repeated 3 times to select for gut-targeting peptide sequence
Methods- Intraluminal Delivery of Phage

30% TBSA steam burn for 7 seconds

balb/c mice

2 hr

30 min

- Perform Laparotomy
- Isolate 3 segments of distal small intestine between silk ties
- Inject 200 μl containing $1 \times 10^9$ phage or control (PBS or “empty phage”)
- Close Abdomen

- Harvest each segment of distal small intestine
- Bowel segments washed with PBS, Trypsin using a peristaltic pump
- Phage DNA isolated from specimens for PCR
### Candidate Peptide Sequences

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>YGFELVMVMSQV</td>
<td>APMITKSWPSGP</td>
</tr>
<tr>
<td>STYAVVTSMSWP</td>
<td>TMSATNTGAMHS</td>
</tr>
<tr>
<td>ASLSGHQYSHTD</td>
<td>TMSATNTGAMHS</td>
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<td>SLPSPHKSQHTW</td>
<td>LPPYLWPSKVTP</td>
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<td>FKPTPGDTTPPS</td>
<td>TGAIPRPGGSLV</td>
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<tr>
<td>NGERTQLRLLL</td>
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<td>HNPMPFPAAQSL</td>
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<td>QLVTSTQPEH</td>
<td>DRNTDIHVSRIP</td>
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<tr>
<td>FSMGIMRPNNL</td>
<td>GTLPIGLTNHK</td>
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<tr>
<td>GFSAPLTHSTP</td>
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<td>SLIAVHSRETAM</td>
<td>MEPHERWVNLHY</td>
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<tr>
<td>QFKGMKDPDPGT</td>
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<tr>
<td>WLAPLPRMAIHT</td>
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</tbody>
</table>

- T-18 identified as candidate gut-targeting sequence
- Isolated in several rounds of screening
Ex Vivo Staining of Intestine

<table>
<thead>
<tr>
<th>T18</th>
<th>AS8</th>
<th>Empty Phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-M13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-m13 + DAPI</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Images show staining patterns with different antibodies and phage treatments.
Intestinal qPCR

Sham Animal

- PBS: 6 particles per mg tissue
- Empty Phage: 4,451 particles per mg tissue
- T18-Phage: 38,591 particles per mg tissue
DNA Sequencing of PCR Product

mRNA: CTT ACT CAT CCT CAG GAT TCG CCG CCG GCT TCT GCG

Protein: L T H P Q D S P P A S A

5’- CGCAGAACCGGCAGCAGAAATCCTGAGGATGATAGTAAG-3’ NS
3’- GCGTCTTCGGCCGCGCTTTAGGACT CCTACTCATTTC-5’ S

12 mer:
Quantum Dots

- Fluorescent nanoparticles

- Emit light which can be visualized using confocal microscopy

- Peptide sequence coupled to Qdot

- Used as a reporter to visualize distribution of the peptide sequence
Qdot Imaging of T18 Sequence

Unconjugated Qdots

Qdots - T18 Conjugate

LYVE-1 (lymph)  Qdots  Overlay

Bar = 50μm
Summary

• Utilized phage display to screen for peptides that target the intestinal barrier

• Identification of a 12 amino acid peptide sequence that binds and internalizes into intestinal epithelial cells after burn injury

• Demonstrated delivery of fluorescent nanoparticles bound to the peptide sequence
Conclusion

• This sequence may allow for targeted therapies designed to attenuate intestinal dysfunction following severe injury, inflammation, or other pathologic conditions of the small bowel
Future #3: The Neuro-Enteric Axis

• Enteric Nervous System
  – Gastrointestinal tissues innervated by complex component of the peripheral nervous system

• Enteric Glia
  – Similar to astrocytes of the CNS
  – Express glial fibrillary acidic protein (GFAP) when activated
  – Promote intestinal barrier function
    • Secretion of S-nitrosoglutathioine (GSNO)
GFAP is required to maintain gut architecture

Sham vs. GFAP Conditional knockout

GFAP-HSV-Tk Mice
- Fatal by 19 days
- Severe inflammation
- Hemorrhagic necrosis

Inflammation activates enteric glia cells

Pro-inflammatory cytokines increase percentage of GFAP positive staining (red) neurons

Addition of enteric glia cells (EGC) to Caco2 cell culture:  
- Increases occludin and ZO-1 levels  
- Improves barrier function (TER and FITC-Dextran)
Enteric glia cells secrete GSNO when activated, which improves intestinal barrier function at low concentrations.

GSNO improves barrier function at low concentrations and increases permeability at high concentrations.
Intestinal GFAP qPCR Time Course

Fold Increase (±SEM)

Sham 2 hour 6 hour 24 hour
Intestinal GFAP- Confocal Time Course

Green = S100
Red = GFAP
GFAP-luc Transgenic Mice

Sham

Burn

2hr

6hr

Gut

Brain
Quantification of Luminescence from GFAP-luc Mice
Histology

Sham  4h Burn  Vagal Stim / Burn  Vagotomy / Vagal Stim / Burn
Intestinal Permeability 4 hrs Post-burn

FITC-Dextran (μg/ml) ± SEM

- Sham: n=4
- Burn: n=5
- Cervical Vagal + Burn: n=5
- Stim: n=5
- Abd Vagotomy Burn: n=5
- Cervical Vagal Abd Vagotomy Stim: n=5
Occludin Western blot 4hrs post-burn
Intestinal GFAP qPCR 4 hours post-burn

Gene Expression: GFAP in Burn Gene study_condition.gxd
GFAP Confocal - 4 hrs post-burn

60X Magnification Comparison

Burn + Vagotomy + Vagal Stim (408)

Burn + Vagal Stim (370)

Sham (368)

Burn (373)
Conclusions

- Past: Translocation through the portal vein to liver.
- Present: Lymph route more important
- Future: Already here
  - Non-invasive method of monitoring organ injury. One animal – multiple measurements
  - Drug delivery to target cells. Specific, more effective, perhaps cheaper
  - Manipulation of PNS and enteric glia – promising therapeutic strategy.

Sham 4hr Burn
The UCSD Team

- **Faculty**
  - R. Coimbra MD, PhD
  - B. Potenza MD
  - J. Doucet MD
  - V. Bansal MD
  - J. Lee MD
  - B. Eliceiri PhD
  - A. Baird PhD
- **TPM**
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Thank you

Downtown San Diego

http://trauma.ucsd.edu