

THE EFFECTS OF LOW LEVEL LASER THERAPY ON INTESTINAL ISCHEMIA  
REPERFUSION INJURY

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL  
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2012

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For Lila May Kirkby

## ACKNOWLEDGMENTS

I would like to thank Dr. Sheilah Robertson for being my committee chair, mentor and an inspiration in life. Thank you for taking this project on and for your dedication to helping me see it through. Thank you to Drs. Freeman, Borsa, Alleman, and Ellison, for your assistance, encouragement, and time commitments to being part of my committee. Thank you to Drs. Hauptman, Reinhardt, Grosche and MacKay for your assistance with sample evaluation. Thank you to Wendy Davies and Jim Burrow for your technical assistance. Thank you to Dr. Dan Lewis for your mentorship, encouragement, support, and most of all belief that I would finish this when others doubted! Thank you Sally O'Connell for all of your help with registration, forms and navigating through this process!

Thank you to my amazing family—Mom, Dad, Mike, Peggy, Tim, Samantha and Aaron—for your support and love! Especially thank you to Aaron for being the most wonderfully positive, encouraging, supportive, kind, and all around fantastic partner I could ask for in life. Thank you to Bailey for the intermittent licks and for just being so good and sweet. Thank you Beans for comedic relief. Finally, in memory of my best friend Bella, you are always with me.

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## LIST OF ABBREVIATIONS

A	Area, in cm <sup>2</sup>
ARDS	Acute respiratory distress syndrome
ATP	Adenosine tri-phosphate
C1	Control group 1: Ischemia and reperfusion, no laser treatment
C2	Control group 2: Laser treatment, no ischemia and reperfusion
COX-2	Cyclo-oxygenase 2
CT	Computed tomography
CW	Continuous wave
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPR	Electron paramagnetic resonance
Fe <sup>3+</sup>	Ferric iron
Fe <sup>2+</sup>	Ferrous iron
GAALAS	Gallium aluminum arsenide
GAAs	Gallium arsenide
GDV	Gastric dilatation and volvulus
HeNe	Helium neon
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HSP	Heat shock protein
Hz	Hertz, pulse frequency
IBD	Inflammatory bowel disease
ICAM 1	Intracellular adhesion molecule 1

I-FABP	Intestinal fatty acid binding protein
IFN $\gamma$	Interferon gamma
IL 1	Interleukin 1
iNOS	Inducible nitric oxide synthase
IPC	Ischemic pre-conditioning
IRI	Ischemia reperfusion injury
J	Joule
kPa	Kilopascal
LDF	Laser doppler flowmetry
LDH	Lactate dehydrogenase
LED	Light emitting diode
LFA	Laser fluorescence angiography
LLLT	Low level laser therapy
MDA	Malodialdehyde
mmHg	Millimeters of mercury
mmol/L	Millimole per liter
MOF	Multiple organ failure
MPO	Myeloperoxidase
mW	Milliwatt
NAC	N-acetylcysteine
NF $\kappa\beta$	Nuclear factor kappa beta
NIRS	Near infrared light spectrophotometry
nm	Nanometer
NO	Nitric oxide
O $_2^-$	Super oxide

$\cdot\text{OH}$	Hydroxyl radical
$\text{ONOO}^-$	Peroxynitrite
P	Power, in W PAF Platelet activating factor
$\text{PCO}_2$	Partial pressure of carbon dioxide
PFC	Perfluorocarbons
PGE-2	Prostaglandin E2
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SMA	Superior mesenteric artery
SOD	Superoxide dismutase
$\text{SpO}_2$	Oxygen saturation of hemoglobin in blood
$\text{StO}_2$	Oxygen saturation in tissue
t	Time, in seconds (s)
TBARS	Thiobarbituric acid reactive substances
$\text{TGF}\beta 1$	Transforming growth factor beta 1
$\text{TNF}\alpha$	Tumor necrosis factor alpha
TPO	Laser treatment following reperfusion
TPR	Laser treatment prior to reperfusion
Tx	Pooled treatment groups; ischemia and reperfusion and laser treatment
VEGF	Vascular endothelial growth factor
VLS	Visible light spectrum
W	Watt
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

8-ISO $\text{PGF}_{2\alpha}$

F2 Isoprostane

$^{99\text{m}}\text{TcO}_4$

Technetium pertechnetate

Abstract of Dissertation Presented to the Graduate School  
of the University of Florida in Partial Fulfillment of the  
Requirements for the Degree of Doctor of Philosophy

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December 2012

Chair: Sheilah Robertson

Major: Veterinary Medical Sciences

Intestinal ischemia reperfusion injury (IRI) in animals and humans is associated with mortality rates between 50-80%. Various pharmacologic agents have been studied as potential treatments for IRI; however, no single treatment has been identified that prevents the damage associated with IRI. Therefore, novel treatment strategies should be sought. Low-level laser therapy is a means of enhancing tissue healing, and can ameliorate IRI in cardiac and skeletal muscle. The objective of this study was to investigate the effects of low level laser therapy (LLLT) in a model of intestinal IRI.

Ninety-six rats were assigned to groups and anesthetized. Small intestinal ischemia was induced by clamping the superior mesenteric artery (SMA) for 60 minutes. A laser diode (70 mW, 650 nm) was applied to the jejunum at a dose of 0.5 J/ cm<sup>2</sup>. Animals were maintained under anesthesia and sacrificed at 0, 1 and 6 hours following reperfusion. Intestinal, lung and liver samples were evaluated histologically. Selected serum cytokine levels were measured using enzyme-linked immunosorbent assay (ELISA).

Intestinal injury was significantly worse ( $p < 0.0001$ ) in animals treated with laser and no IRI compared to sham. Intestinal injury was significantly worse in animals that

underwent IRI and laser treatment at all time points compared to sham ( $p < 0.001$ ). In animals that underwent IRI, those treated with laser had significantly worse intestinal injury compared to those that did not have laser treatment at 0 ( $p = 0.0104$ ) and 1 ( $p = 0.0015$ ) hour of reperfusion. After 6 hours of reperfusion there was no significant difference in injury between these two groups. Lung injury was significantly decreased following IRI in laser-treatment groups ( $p < 0.001$ ). Intestinal IRI resulted in significantly increased serum tumor necrosis factor (TNF) alpha, interleukin (IL)-6 and IL-10 ( $p < 0.05$ ). Laser treatment of intestinal IRI did not significantly affect the expression of these cytokines. Laser treatment to uninjured bowel did not increase the expression of the measured cytokines.

In conclusion, at the dose and parameters used, LLLT did not protect against intestinal IRI in the acute phase of injury. However, laser did provide protection against distant organ injury and this may be partially mediated by TNF alpha and IL-6.

# CHAPTER 1

## INTESTINAL ISCHEMIA REPERFUSION INJURY IN VETERINARY PATIENTS

### **Mechanism of Action**

Ischemia reperfusion injury (IRI) is a sequence of events with potentially devastating consequences at the site of injury and at distant organs.<sup>1-4,7-11</sup> The process begins when blood supply, and therefore oxygen delivery, to an organ is disrupted.<sup>2</sup> This can occur through various mechanisms including intravascular obstruction (thromboembolism), extraluminal compression (vessel strangulation), severe hypotension (septic shock), and organ transplantation.<sup>2-4</sup> Warm ischemia describes events that occur within the body whereas cold ischemia refers to the controlled disruption of blood flow that occurs during organ transplantation.<sup>3</sup> This research will focus on warm ischemia, particularly as it pertains to veterinary patients.

### **Ischemia**

Cells rely on a constant supply of oxygen for cellular respiration, metabolism, and normal function.<sup>1-4</sup> In periods of hypoxia, cells are unable to generate sufficient (adenosine tri-phosphate) ATP, and existing ATP is degraded into adenosine and then hypoxanthine. Concurrent with ATP catabolism is the generation of lactate and hydrogen ions through anaerobic metabolism, resulting in decreased intracellular pH.<sup>1-4</sup> Many intracellular regulatory proteins and enzymes are damaged in this environment leading to further disruption of cell function. For example, iron is released from ferritin, resulting in the accumulation of free intracellular iron.<sup>1,3</sup> ATP-dependent cell membrane pumps become inactive leading to dysregulation of transmembrane ion flow. This results in a net cellular efflux of potassium and an influx of sodium, calcium and chloride into the cell. The consequence of this ion flux is cellular swelling and ineffective

membrane function leading to leakage of proteolytic and lysosomal enzymes from the cell.<sup>1-4</sup>

Increased intra-cellular calcium activates a protease that catalyzes conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO).<sup>1-4</sup> Xanthine dehydrogenase and XO can both convert hypoxanthine to uric acid, with the former being oxygen independent and XO requiring oxygen.<sup>1-4</sup> In ischemia the XO form predominates, so hypoxanthine accumulates and will become an important factor when oxygen supply is re-established. Increased cytosolic calcium also leads to further series of chemical reactions that can culminate in cell apoptosis or necrosis.<sup>1-4</sup>

Nitric oxide (NO) is an important regulator of endothelial cell function and vascular tone, and NO production and activity are limited during ischemia, resulting in vasoconstriction.<sup>1,3</sup> The arachidonic acid cascade is disrupted, contributing to further vasoconstriction and platelet clumping.<sup>1,3</sup> Furthermore, endothelin, a potent vasoconstrictor, is upregulated in the endothelium during ischemia.<sup>1,3</sup>

The inflammatory cascade is initiated during ischemia through activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B).<sup>1</sup> Intracellular adhesion molecule-1 (ICAM 1) and E-selectin are up-regulated which, like hypoxanthine, will become important during reperfusion.<sup>1</sup> Finally, the complement cascade and platelet activating factor (PAF) are activated, setting the stage for continued dysfunction once blood flow is re-established.<sup>1,3</sup>

## **Reperfusion**

With continued ischemia, irreversible cell damage and cell death can occur. However, even if ischemic injury alone is not lethal, when blood flow to ischemic tissue is restored, generation of reactive oxygen species (ROS) can result in even greater

damage to local and distant tissue than that which occurred due to ischemia.<sup>1-4</sup> The process of reperfusion injury begins when oxygen combines with XO to convert accumulated hypoxanthine to uric acid, with concurrent generation of the superoxide radical ( $O_2^-$ ).<sup>1-4</sup> Superoxide is a relatively innocuous ROS, but does have the capacity to increase free intracellular iron and convert ferric ( $Fe^{3+}$ ) to ferrous ( $Fe^{2+}$ ) iron.<sup>1-4</sup> Superoxide is converted to hydrogen peroxide ( $H_2O_2$ ) by superoxide dismutase (SOD). Free  $Fe^{2+}$  reacts with  $H_2O_2$  to form the highly reactive hydroxyl radical ( $\cdot OH$ ) through the Haber-Weiss reaction.<sup>1</sup> The hydroxyl radical is a very potent ROS and when it reacts with other molecules a chain reaction of destructive events occurs. These include deoxyribonucleic acid (DNA) damage, cellular enzyme and protein degradation, and lipid peroxidation that leads to cell membrane permeability.<sup>1,3</sup>

During ischemia NO production is decreased but the enzyme inducible nitric oxide synthase (iNOS) is upregulated.<sup>1</sup> Nitric oxide has the potential to be both beneficial and harmful to tissues during IRI. When reperfusion occurs, a large amount of NO may initially be generated; however, NO reacts with  $O_2^-$  to form the very potent free radical, peroxynitrite ( $ONOO^-$ ).<sup>1</sup> Peroxynitrite can cause substantial damage to cell membranes, DNA, and cellular proteins.<sup>1</sup> Moreover, the depletion of NO through this reaction leads to further endothelial cell dysfunction, increased expression of adhesion molecules, and increased cell and tissue permeability.<sup>1</sup>

In addition to ROS and NO, neutrophils are an important mediator of IRI.<sup>3,12</sup> During ischemia, ligands such as ICAM-1 and E- and P- selectins are expressed on endothelial cell membranes associated with the up-regulation of NF- $\kappa$ B.<sup>1,12</sup> During reperfusion, neutrophils adhere to these endothelial surface molecules, undergo

diapedesis, and initiate further chemotaxis of additional leukocytes.<sup>1,12</sup> Furthermore, inflammatory mediators such as tumor necrosis factor alpha (TNF  $\alpha$ ), interleukin (IL)-1 $\beta$ , PAF and complement, which are all potent chemottractants, are produced during IRI and contribute to further sequestration of leukocytes at the site of injury.<sup>1,12-15</sup> Neutrophils have the capacity to generate ROS during their respiratory burst, which along with the release of proteolytic enzymes such as myeloperoxidase, contribute to further tissue injury and obstruction of the microvasculature.<sup>1,12</sup>

The “no-reflow” phenomenon describes the decrease in blood flow to a previously ischemic area following reperfusion.<sup>7</sup> This condition develops as a consequence of endothelial cell swelling and sloughing, vasoconstriction, and erythrocyte, platelet and neutrophil aggregation within the microvasculature.<sup>7</sup>

### **Intestinal IRI**

Oxygen delivery to cells depends on both blood flow (which is determined by heart rate, blood volume and vascular resistance or patency) and the oxygen content of the blood (which is determined primarily by the oxygen saturation of hemoglobin and also the amount of dissolved oxygen in blood).<sup>16</sup> Hypoxia refers to a state of decreased oxygen availability either to the entire body or localized to an organ or tissue, or in other words, the oxygen demand of the tissue exceeds the availability. Causes of hypoxia include alterations in blood flow (cardiac arrhythmias, hypovolemia, hypo or hypertension and vascular occlusion) or disturbances of blood oxygen content (pulmonary disease, anemia). Anoxia is a state of complete deprivation of oxygen supply. Ischemia is a restriction of blood flow to an organ or tissue and can cause either hypoxia or anoxia depending on the degree of vascular occlusion.<sup>4,16</sup>

Among all visceral organs, the intestines are considered to be the most sensitive to IRI.<sup>1,15</sup> The small intestinal mucosa is particularly sensitive to the effects of IRI, especially the tips of the villi.<sup>4,7,17</sup> This can be attributed to the unique anatomy of the intestinal microcirculation, the inherent susceptibility of differentiated, mature enterocytes to hypoxia, the effects of shear stress on the tips of the villi, and the action of intraluminal pancreatic enzymes.<sup>4,7,17</sup>

The intestines receive 20-35% of cardiac output, and 70% of this blood flow is directed toward the intestinal mucosa and submucosa.<sup>7</sup> Similar to the kidneys and brain, myogenic and metabolic auto-regulatory mechanisms are in place to maintain a consistent blood flow despite fluctuation of systemic blood pressure and sympathetic activity.<sup>4,7</sup> Furthermore, the organization of the vasculature is specialized within the intestine in order to maintain a constant level of oxygen uptake to support digestion and host defenses.<sup>4</sup> A vascular plexus within the submucosa gives rise to central arterioles that course within the villi and form a mesh-like subepithelial capillary plexus at the villous tip.<sup>4</sup> Venules course parallel to these arterioles allowing for countercurrent blood flow to occur, which accounts for part of the autoregulatory hemodynamics.<sup>4</sup> That is, during periods of intestinal “rest”, a portion of arteriolar oxygen is shunted to venules near the crypts without reaching the villous tips, and during the post-prandial state, greater amounts of oxygen are supplied to the tips.<sup>4</sup> If blood flow is reduced up to 50%, auto-regulation enables oxygen redistribution and increased oxygen extraction from highly metabolic cells at the villous tips.<sup>4</sup> However, this mechanism can exacerbate the low-flow state: If significant hypotension (<40 mmHg) or ischemia (>50% reduction in blood flow) occurs, the blood flow velocity through the villi is decreased to the point

where oxygen “short-circuits” and diffuses between arteriole and venule at the crypts and the tips become anoxic.<sup>4,7</sup>

The intestinal mucosa normally undergoes regular cell turn over and migration of cells from the crypts to the villus tips.<sup>18</sup> As enterocytes migrate toward the tip they undergo differentiation from a primarily secretory cell to one with absorptive capabilities.<sup>18</sup> A study in rats found that differentiated enterocytes were significantly more susceptible to IRI compared to undifferentiated enterocytes following 30-60 minutes of ischemia and up to 4 days of reperfusion.<sup>19</sup>

Another mechanism of mucosal injury in IRI is the mechanical shear stress placed on the villi tips by luminal contents.<sup>3</sup> The first morphological change that occurs in the mucosal epithelium is the generation of Gruenhagen’s Space or accumulation of fluid within the subepithelial space.<sup>20</sup> Once this occurs, epithelial cells can easily be detached and sloughed off into the intestinal lumen.<sup>20</sup> Intraluminal pancreatic endoproteases can also contribute to proteolytic destruction of the mucosa, and studies have shown that prior ligation of the pancreatic duct can attenuate intestinal IRI.<sup>21,22</sup>

A sequence of events occur following ischemic insult to the bowel that begins with an increase in capillary permeability, followed by increased mucosal permeability, superficial mucosal damage and cell sloughing, transmucosal damage, and finally transmural injury.<sup>4,23</sup> These processes can be observed histologically and grading systems have been described to document these findings.<sup>20,23</sup>

### **Consequences of Intestinal IRI**

The consequences of intestinal IRI are related to the duration and extent of ischemia (low-flow or partial vs. complete, arterial alone vs. arterial and venous).<sup>4,20,23,24,25</sup> Complete intestinal ischemia is more devastating than incomplete;

however, paradoxically, partial ischemia has been reported to result in more substantial reperfusion injury compared to complete ischemia.<sup>4,23,24,25</sup> For example, Megison et al. compared rat models that included partial intestinal ischemia (superior mesenteric artery [SMA] occlusion only) and complete ischemia (SMA occlusion plus collateral ligation) over various time periods.<sup>24</sup> These investigators found that complete occlusion resulted in greater mortality compared to partial ischemia and that longer ischemia times (up to 90 minutes) were more devastating than shorter ischemic periods.<sup>24</sup>

Park et al. compared occlusion of the intestinal arterial and venous supply to arterial occlusion alone.<sup>23</sup> This group found that arterial and venous occlusion for less than 20 minutes did not result in detectable tissue damage. Ischemia times between 20 and 90 minutes led to mucosal villous injury with a direct relationship between ischemia time and extent of injury; however, reperfusion did not result in perpetuation of injury regardless of the duration of ischemia. Conversely, when only the arteries were clamped for 40 to 60 minutes, reperfusion caused statistically significant exacerbation of mucosal injury.<sup>23</sup>

Another model of intestinal IRI is low-flow ischemic injury, which aims to mimic conditions such as severe intestinal distention and damage that may occur at the periphery of strangulated bowel.<sup>20,26-29</sup> Chiu et al. compared different rates of SMA blood flow in a dog model and found that repeatable mucosal damage occurred when blood flow was decreased to 30% of baseline.<sup>20</sup> Intestinal damage was even more severe and developed earlier if the SMA was completely occluded. Reduction of intestinal blood flow to 20% of baseline by partial arterial occlusion is a common model used in studying equine intestinal IRI.<sup>26-29</sup> This model can maintain systemic

hemodynamic and metabolic stability while predictably causing alterations in intestinal metabolism and mucosal injury.<sup>28,29</sup> Moore et al. used this model to compare 6 hours of low-flow ischemia to 3 hours of low-flow ischemia followed by 3 hours of reperfusion and found that ischemia and reperfusion caused more substantial mucosal injury than ischemia alone.<sup>29</sup>

The conclusions from these studies and others is that if ischemia is not severe enough to cause detectable mucosal injury, then reperfusion is unlikely to cause notable additional damage. However, if ischemia causes severe mucosal injury, further damage will occur with reperfusion. If ischemic injury is severe enough to affect the full thickness of bowel wall, reperfusion does not seem to result in further observable injury, although bowel necrosis and perforation may be imminent. Thus, there appears to be a “reperfusion injury window” during which time intervention following ischemia may reduce further mucosal injury.

The untoward physiologic consequences of intestinal IRI can be appreciated both in local and distant tissue.<sup>1-4,7-11</sup> Damage of intestinal villi causes a decrease or loss of absorptive function, which can lead to malabsorption of nutrients, and chronic metabolic derangement.<sup>1-4</sup> Alterations in intestinal permeability can cause bacteria and endotoxin translocation across the gut wall and subsequent dissemination throughout the body leading to sepsis, endotoxemia and multiple organ failure (MOF).<sup>1,30,31</sup>

One of the most important consequences of intestinal IRI is the development of secondary organ injury.<sup>7-11</sup> Once blood flow is reestablished, ROS and inflammatory cytokines that were generated during IRI are transported throughout the body. The myocardium and lungs are particularly sensitive to these molecules.<sup>8-11</sup> Myocardial

contractility can become impaired within 2 hours of intestinal ischemia and reperfusion, and this change may persist for up to 16 hours in rats.<sup>32</sup>

Pulmonary infiltration of neutrophils and acute respiratory distress syndrome (ARDS) is well documented following intestinal IRI.<sup>1-4,9-11</sup> More specifically, the delivery of ROS and inflammatory toxins to the lungs leads to increased pulmonary microvascular permeability, alveolar endothelial cell injury, depletion of lung ATP, and accumulation of leukocytes within the interstitial spaces.<sup>3,9-11</sup> Intestinal and alveolar endothelial cell damage exposes subendothelial collagen, leading to activation of the intrinsic coagulation cascade. The extrinsic cascade is also activated by the release of tissue factor from injured tissue.<sup>3</sup> Activation of both coagulation pathways results in initial hypercoagulability, associated thrombotic events and further tissue injury, and is often followed by disseminated intravascular coagulation (DIC), systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF).<sup>3,30,31</sup>

### **Intestinal Healing**

The intestinal mucosal surface is comprised of a monolayer of columnar epithelial cells with other specialized cells (Goblet, Paneth) intermixed.<sup>18</sup> Epithelial migration from the villous crypts to tips with subsequent apoptosis or shedding into the lumen is a normal physiologic function and cell turn over occurs every 2-5 days.<sup>18</sup> As such, the intestinal mucosa has an inherent capacity for rapid healing with focus placed on maintaining the barrier function of the epithelial lining.

Intestinal mucosal injury can occur due to inflammatory, ischemic, or intraluminal toxic insults or through normal digestive processes. The first step following injury begins within minutes to hours following insult and is termed epithelial restitution.<sup>18</sup> In this process, epithelial cells surrounding the injury change shape from a columnar to a

more flattened cell and migrate into the denuded space in order to reestablish the mucosal barrier. The next step is proliferation of enterocytes within the crypts, which begins hours to days after injury.<sup>18</sup> Finally, enterocytes undergo differentiation and maturation in order to restore normal mucosal function.<sup>18</sup>

As intestinal healing pertains to IRI, the capacity for mucosal recovery depends on the severity and duration of ischemia. The intestinal mucosa can potentially recover within 24 hours after ischemic injury.<sup>4,17,25</sup> Park et al. demonstrated in a rat model (SMA occlusion) that after 45 minutes of ischemia, mucosal repair was evident within 3 hours, whereas 90 minutes of ischemia required 18 hours before mucosa repaired. After 24 hours, restitution of the mucosal surface was complete.<sup>25</sup> Toth et al. performed a study that chronicled intestinal recovery following 60 minutes of SMA occlusion and 1 hour, 24 hours and 30 days of reperfusion in a rat model.<sup>17</sup> This group found that at 1 hour after ischemia, damage to the intestinal epithelium included lifting of the epithelial layer, villi disintegration, crypt layer destruction and transmural infarction. However, after 24 hours of reperfusion, there was no significant difference between injured rats and sham/control rats. After 30 days there were increased numbers of Paneth cells in previously injured rats compared to control rats but no significant difference in other histological parameters.<sup>17</sup>

The intestine's capacity for complete healing following ischemia underscores the idea of a "therapeutic window" which may allow for intervention that not only hastens intestinal recovery but also attenuates distant reperfusion injury.

## Comparative Clinical Aspects of Intestinal IRI

### Canine and Feline

Intestinal IRI can occur in dogs and cats due to occlusive and non-occlusive conditions. Intestinal or mesenteric volvulus (and much more commonly gastric distension and volvulus [GDV]), intraluminal obstruction (foreign body, tumor), strangulating hernia, mesenteric or jejunal arterial thrombosis and portal vein thrombosis are potential causes of occlusive ischemia.<sup>33-42</sup> Low flow states such as heart failure, trauma and shock are associated with non-occlusive intestinal ischemia.<sup>30,31</sup> Portal hypertension, a potential complication following portosystemic shunt ligation, can also lead to intestinal IRI.<sup>33</sup>

An historic study by Chiu et al., published in 1972, described a canine experimental model of small intestinal IRI by occlusion of the cranial mesenteric artery.<sup>42</sup> This group sought to investigate whether the devastating systemic consequences of intestinal ischemia were due to hypovolemic shock versus the release of “toxic substances” into the circulation. They found an 89% mortality rate in dogs that underwent 3 hours of ischemia followed by reperfusion without any further intervention, whereas the mortality rate was decreased to 36% when immediate fluid therapy was instituted and continued for 24-48 hours.<sup>42</sup> Another group of experimental dogs did not have release of the mesenteric artery occlusion but rather, the artery was flushed with crystalloid fluid to allow portal absorption of accumulated “toxic factor”. Dogs in this group did not show evidence of circulatory collapse over the following 12 hours, prior to euthanasia. These authors concluded that while the absorption of “intestinal toxic factors” may play a role in death from intestinal IRI, a more important cause of mortality

is circulatory collapse and shock due to loss of large volumes of fluid and albumin into the intestinal lumen.<sup>42</sup>

The most common cause for occlusive small intestinal ischemia in dogs and cats is luminal obstruction by a foreign body, leading to compression of the microvasculature and potentially full-thickness bowel injury.<sup>40</sup> Surgical intervention involves removal of the offending foreign body (or occasionally tumor) along with any non-viable bowel.<sup>40</sup>

Another common cause of enteric IRI in dogs is GDV.<sup>8,34,38,41</sup> This condition is a medical and surgical emergency and is associated with a high mortality rate when intervention is delayed.<sup>8,34,38,41</sup> In treating this condition the stomach is decompressed and repositioned in a normal anatomic location. Reperfusion of the organ occurs quickly and systemic consequences, including cardiac dysrhythmias and pulmonary edema, can occur within minutes to hours. Volume resuscitation prior to, during and following surgery is crucial in order to maximize intestinal perfusion and minimize cardiovascular collapse.<sup>8,34,38,41</sup>

Surgical decision making becomes challenging when a significantly large section of stomach or intestine is affected and/ or the viability of the bowel is questionable. The study by Chiu et al. discussed above highlights the importance of aggressive medical management in combination with any potential surgical intervention.

Septicemia/endotoxemia is a relatively common clinical scenario in small animal patients following trauma and hemorrhagic shock.<sup>30,31</sup> Shock leads to reduced intestinal perfusion, and the sequelae of IRI and mucosal injury progresses, with loss of mucosal barrier function and translocation of luminal bacteria, endotoxin, and inflammatory cytokines.<sup>30,31</sup> Bacteria become sequestered in intestinal lymphatics and are not

commonly cultured from the blood stream or other tissues; however, dissemination of inflammatory products and ROS to the lungs, heart and other organs results in SIRS, MOD, DIC and potentially death.<sup>30,31</sup> Recognition of this pathophysiologic consequence of trauma and shock is essential to provide appropriate intervention and mitigation of intestinal ischemia.

## **Equine**

The most common causes of intestinal IRI in the horse are volvulus or incarceration of the small intestine and volvulus of the large colon.<sup>3</sup> These conditions result in strangulation obstruction of arterial and venous blood flow to the associated bowel. Once ischemia occurs, mucosal epithelial cells become completely denuded by 3 hours and villi contract to the level of the crypts. If ischemia persists up to 5 hours, complete mucosal necrosis occurs and after 7 hours of ischemia, necrosis extends beyond the muscularis layer.<sup>43,44</sup> It has been shown that 3-4 hours of complete ischemia causes irreversible damage to the large colon.<sup>44</sup>

Surgical intervention is indicated to relieve the vascular obstruction and to remove non-viable intestine. However, even with surgery, the prognosis for long-term survival is guarded. One study found that horses with small intestine strangulating lesions requiring resection and anastomosis had approximately 10% survival at 3 years.<sup>45</sup> Horses with large colon volvulus have reported survival rates between 20-40%.<sup>3</sup> The overall mortality rate for horses affected by strangulating obstruction of the intestine is 50-80%.<sup>3</sup> Ultimately, the prognosis will depend on the amount of intestine damaged and feasibility of removing all effected tissue. Aggressive medical management is necessary in addition to surgery in order to address hypovolemia, endotoxemia and consequences of distant organ reperfusion injury.<sup>3,30,31</sup>

## **Human**

Compared to dogs and horses, intestinal ischemia in humans is less common yet can occur due to a wider range of causes.<sup>7,46-48</sup> Conditions associated with intestinal IRI in human patients can be divided into acute and chronic and occlusive and non-occlusive disease processes. Acute intestinal or mesenteric ischemia can occur due to vascular occlusive conditions such as: arterial or venous thromboembolism, following vascular surgery, trauma, septic and hypovolemic shock, heart failure, cardiopulmonary bypass, intestinal transplant, strangulated hernias, small bowel volvulus, and neonatal necrotizing enterocolitis.<sup>46-48</sup> Chronic conditions that lead to intestinal IRI include: atherosclerosis, fibromuscular dysplasia, inflammatory bowel disease (IBD), radiation injury, and congenital malformation.<sup>48</sup> Primary vascular conditions such as atherosclerosis, intestinal transplantation and vascular surgery have not been recognized as causes of intestinal IRI in clinical veterinary patients.

Regardless of the underlying cause, intestinal ischemia is considered a life-threatening emergency and the prognosis depends on rapid, accurate diagnosis and intervention. The frequency of intestinal ischemia is low compared to other indications for laparotomy and diagnosis of the condition can be challenging.<sup>7,46,48</sup> Initial presenting signs may be non-specific and physicians must consider bowel ischemia in their differential list for abdominal discomfort since delayed diagnosis and treatment can result in bowel necrosis. The overall mortality rate for human patients with intestinal ischemic conditions ranges between 60-80%.<sup>7,46</sup>

### **Diagnosis of Intestinal Ischemia**

Accurate and timely diagnosis of intestinal ischemia is crucial for successful intervention and optimal outcomes. There appears to be a therapeutic window following

significant ischemia during which time the deleterious effects of reperfusion can be mitigated--salvaging the affected bowel and preventing secondary organ injury. In humans it is believed that intestinal ischemia lasting less than 6-8 hours causes damage that is entirely reversible.<sup>48</sup> A similar “golden period” has not been specifically reported in clinical veterinary patients, but is likely a similar time frame. However, diagnosis of this critical phase is quite challenging, even at the time of surgery and inspection of the bowel since significant mucosal damage may be present despite a grossly viable serosal surface. Numerous techniques to determine intestinal viability have been employed in clinical and experimental settings and the pros and cons of the most commonly used techniques will be outlined below.<sup>38,39,48-62</sup>

## **Clinical: Pre-operative**

### **Ultrasound**

Ultrasound is a first line screening tool used in humans to diagnose chronic intestinal ischemia by evaluating arterial blood flow velocity.<sup>48</sup> Values have been established in humans that correlate with vessel stenosis and intestinal ischemia; however, accuracy is highly operator dependent. There is one report in the small animal veterinary literature that associates the ultrasonographic finding of corrugated small intestine with bowel infarction.<sup>39</sup>

### **Angiography**

Digital subtraction angiography is considered the gold standard in humans for diagnosis of vessel stenosis.<sup>48</sup> It is particularly beneficial because intra-vascular procedures can be performed at the same time.<sup>48</sup> At this time there are no reports of angiography for the diagnosis of intestinal ischemic conditions in veterinary patients and

this modality is less useful in animals due to the paucity of naturally occurring intravascular stenotic diseases.

### **Computed tomography angiography**

Computed tomography (CT) with angiography may be the ideal modality for diagnosing acute intestinal ischemic conditions in humans since it can demonstrate vessel stenosis as well as bowel wall thickness, position, distention, free peritoneal air, or other alternative conditions that present with similar symptoms.<sup>48</sup> A multi-slice CT with <2 mm slice thickness is required for accurate evaluation of small vessels.<sup>48</sup> At this time there are no reports of using this modality in clinical veterinary patients; however, as this technology becomes more readily available, CT angiography may prove useful, particularly for small animal acute abdominal conditions.

### **Magnetic resonance angiography**

This modality has been described for use in humans to diagnose chronic bowel ischemia and has the potential for excellent sensitivity and specificity but is highly operator dependent.<sup>48</sup> There are no reports of its use in veterinary patients for this purpose.

### **Endoscopy**

Endoscopy is commonly performed in human patients as part of diagnostic work up in cases of chronic small intestinal ischemia and for colonic ischemia. Drawbacks of this diagnostic modality include potential for missing patchy areas of ischemia and the affected area may not be accessible by the endoscope.<sup>48</sup>

### **Tonometry**

Intra-gastric (or intra-intestinal) partial pressure of carbon dioxide (pCO<sub>2</sub>) can be measured using a tonometry catheter either pre-, intra-, or post-operatively.<sup>48</sup> During

bowel ischemia there is an increased production of acids due to anaerobic metabolism. These acids are buffered locally by bicarbonate, resulting in increased pCO<sub>2</sub> in the tissue. The most specific marker of ischemia is documentation of an increased pCO<sub>2</sub> gradient between luminal and arterial CO<sub>2</sub>.<sup>48</sup> The pCO<sub>2</sub> gradient will remain normal (< 11 kilopascal (kPa) or 60 millimeters of mercury [mmHg]) until blood flow is <50%, at which point it will begin to decrease sharply.<sup>48</sup> This holds true for all models of ischemia and in all animals.<sup>48</sup> Nasogastric tonometry is performed commonly in humans but is not routinely available for veterinary patients.

### **Blood and peritoneal biochemical alterations**

Phosphate and potassium are often elevated in the serum and peritoneal fluid of horses and dogs with intestinal IRI.<sup>28,50,51</sup> Phosphate elevation in the blood and peritoneal fluid has been correlated to the extent of intestinal IRI in horses, and higher levels are associated with non-viable bowel and euthanasia.<sup>50</sup> Lactate is also elevated in the blood and peritoneal fluid in response to ischemic injury.<sup>28</sup> In dogs, a serum lactate of >6.6 millimole per liter (mmol/L) has been significantly associated with gastric necrosis in clinical cases of GDV.<sup>52</sup> Blood lactate also increases significantly in horses with intestinal IRI.<sup>28</sup>

### **Clinical: Intra-operative**

#### **Gross inspection**

The most commonly used intra-operative technique for evaluating bowel viability is subjective assessment of stomach or intestinal wall thickness, serosal color, presence of peristalsis, and serosal capillary perfusion/ blanching.<sup>38</sup> An experimental model of canine GDV found subjective assessment of stomach wall viability to have an accuracy of 85%.<sup>53</sup> A prospective clinical study in human patients with acute mesenteric

infarction found that clinical judgment of bowel viability had an 87% accuracy compared to 100% accuracy and sensitivity using laser doppler.<sup>53</sup> There is certainly a relationship between surgeon experience and interpretation of subjective findings, and failure to appropriately address non-viable tissue can lead to increased morbidity and mortality.

### **Laser doppler flowmetry (LDF)**

Laser doppler flowmetry has been used in experimental and clinical settings and is a reproducible, quantitative, and relatively accessible modality that can be utilized at the time of surgery.<sup>54-58</sup> This technique has been shown to accurately detect changes in capillary blood flow in the stomach of dogs in an experimental model of GDV.<sup>38</sup> In humans, LDF has been shown to be 100% sensitive and 93% specific for intestinal viability when used intra-operatively.<sup>55</sup> Laser doppler flowmetry functions using a laser beam (monochromatic, coherent light) that can penetrate approximately 6mm.<sup>38</sup> The laser probe is placed over a sample of tissue and measures the capillary blood flow beneath. Since only the area beneath the probe is being sampled, several locations throughout affected tissue should be tested.

Early studies using ultrasound doppler did not find accurate detection of intestinal ischemia, particularly in instances of venous occlusion.<sup>60,61</sup> The primary difference between ultrasound and laser doppler techniques is that ultrasound waves are longer than laser waves and accuracy is limited when target tissue is located within millimeters of the probe. This has resulted in a high rate of false-negatives and false-positives in experimental studies.<sup>59,60</sup> Laser doppler is preferred over ultrasound doppler for clinical and experimental evaluation of bowel viability.<sup>38,54-61</sup>

## **Fluorescein**

Intravenous injection of fluorescein dye has been used in human and animal studies to differentiate viable from non-viable bowel by exposing the serosal surface to ultraviolet light.<sup>59-62</sup> This technique can be used during laparotomy or laparoscopy; however the accuracy is considered relatively low. In particular, fluorescein studies may over-estimate the affected area when there is venous but not arterial occlusion, resulting in excessive tissue resection.<sup>59</sup> A dog model of GDV reported the accuracy for predicting non-vital stomach wall to be 58%.<sup>53</sup>

## **Laser fluorescence angiography (LFA)**

Intravenous injection of the fluorescent dye indocyanine green and subsequent laser illumination of the bowel is a relatively new, yet reliable and repeatable method of assessing tissue perfusion.<sup>59</sup> In studies of human patients undergoing intestinal resection and anastomosis, intra-operative LFA has been shown to reduce the risk of anastomotic leakage due to non-viable anastomotic margins by 60-84%.<sup>64</sup> Limitations of this diagnostic technique include interference by intraluminal contents (therefore unlikely to be useful in acute or emergency situations) and no current recommendations for LFA values that represent irreversible necrosis.<sup>59,64</sup> This modality has been studied in animal models but not in clinical veterinary patients.

## **Pulse oximetry**

Calculation of arterial oxygen saturation using pulse oximetry is a useful clinical method for evaluating systemic arterial hemoglobin saturation and has been investigated as a tool for assessing bowel viability.<sup>58,59</sup> Oxygen saturation (SpO<sub>2</sub>) is calculated by measuring the difference in light absorption between oxygen bound and unbound hemoglobin. While there is literature describing the value of pulse oximetry in

experimental animal models and human case reports of intestinal ischemia, this modality does not actually measure blood flow or tissue viability and has been associated with a high rate of false negative and false positive assessments.<sup>59</sup> Thus at best, pulse oximetry can only be used as an adjunct to other methods of assessing bowel integrity.

### **Light spectrophotometry**

Tissue oxygen saturation (StO<sub>2</sub>) can be measured using either visible (VLS) or near-infrared light spectrophotometry (NIRS) and both techniques have shown value in human clinical studies of intestinal ischemia.<sup>59</sup> Advantages of VLS include the narrow range of normal values (high specificity), relatively shallow depth of light penetration (2 millimeters) resulting in accurate assessment of bowel wall StO<sub>2</sub>, and ability to provide readings without direct contact with tissue. The primary advantage of NIRS is a larger volume of tissue sampled including evaluation of arterial, venous and capillary StO<sub>2</sub> (versus only capillary assessment with VLS).<sup>59</sup> While these techniques are promising, there are currently limitations including lack of standardized equipment and therefore readings, and likewise, specific guidelines for clinical decision making based on StO<sub>2</sub> results have not been established. Furthermore, intraluminal intestinal contents, including bile, stool, and food can interfere with oximetry reading.

### **Scintigraphy**

Nuclear imaging using technetium pertechnetate (99mTcO<sub>4</sub>) was accurate in detecting ischemic areas of the stomach in 91% dogs in a GDV model and 79% accurate with clinical GDV.<sup>64,65</sup> This modality, however, does not provide specific anatomic landmarks, is not readily available in most clinical settings, and the radiation exposure to the patient and attending medical staff is not ideal.

## **Research and Laboratory Techniques**

### **Colored microspheres**

Intravenous injection of colored microspheres followed by measurement of their presence in tissue is considered the gold standard for assessing capillary blood flow.<sup>38,66</sup> This is not a clinically applicable technique but is employed in the experimental setting particularly for validation of novel diagnostic techniques.

### **Serum biomarkers**

D-lactate, lactate dehydrogenase (LDH) and i-FABP (intestinal fatty acid binding protein) have been shown in animal models to be an early marker of intestinal ischemia.<sup>67,68</sup> i-FABP is an enzyme present in mature enterocytes at the tips of intestinal villi and is released early after ischemic injury to the villi. However, these biomarkers have not been sensitive or specific for intestinal IRI in humans as they can also be elevated with pancreatitis or IBD.<sup>67</sup> Likewise, measurement of these markers is not readily available for use in veterinary clinical cases.

### **Histology**

Histologic examination of tissue is the ultimate gold standard for assessing damage that reflects ischemia and reperfusion but is not always clinically applicable.<sup>55</sup> Histology is used in experimental models to describe the degree of intestinal injury or on post-mortem examination to determine cause of death. The use of intra-operative frozen sections can accurately predict survival in horses clinically effected by large colon torsion; however, limitations of this procedure include the need for specialized equipment, availability of a pathologist at the time of surgery, and the potential for non-representative samples.<sup>69</sup>

## **Thiobarbituric acid reactive substances (TBARS)**

Reactive oxygen species commonly attack lipids, resulting in breakdown products such as malodialdehyde (MDA), conjugated dienes, short-chain alkanes, and lipid hydroperoxides. The TBARS test is the most frequent analysis used to measure MDA concentrations in serum or tissue.<sup>49</sup> The down side of this test is that 98% of MDA that reacts with TBARS is formed after collection, so sensitivity and specificity can be inconsistent.<sup>49</sup> When serum or plasma is used for TBARS, MDA can form secondary to platelet activation and thromboxane synthesis. Sample handling and testing is very important in order to obtain the highest specificity. MDA can also be measured using enzyme-linked immunosorbent assay (ELISA), and this newer technique may become more readily available and preferred compared to TBARS.<sup>49</sup>

## **Isoprostanes**

When ROS damage cell membrane, arachadonic acids are broken down and isoprostanes are formed. F2-isoprostane (8-isoPGF<sub>2</sub>α) has been shown to be a reliable and non-invasive way to measure lipid peroxidation in vivo using tissue or fluid, particularly urine, samples.<sup>49</sup> However, this test is not specific for intestinal IRI.

## **Measurement of ROS**

Oxygen radicals are very difficult to measure because of their brief half-life and high reactivity. In experimental settings they can be measured using electron paramagnetic resonance (EPR) spectroscopy.<sup>49</sup>

## **Inflammatory markers**

Serum and tissue elevation of inflammatory products such as TNF-α, IL-1, and IL-6 can be indicative of ischemic injury. These markers are commonly employed to document systemic consequences of intestinal IRI.<sup>9-11,49</sup> Neutrophil sequestration in

tissue can be measured using myeloperoxidase (MPO) activity and calprotectin.<sup>12,70</sup> Both of these tests are commonly used in experimental studies of IRI.

### **Treatment**

Many methods of treating intestinal IRI have been investigated, yet the optimal intervention has yet to be found or perfected.<sup>1,49</sup> Treatments can be directed toward various steps within the IRI cascade including: reducing ROS production, increasing ROS scavenging, suppressing inflammation, preventing expression of adhesion molecules and leukocyte adhesion, modulation of vascular tone and intravascular coagulation, attenuating interstitial edema formation, enhancing local blood flow and oxygen delivery, and improving tissue healing capacity.<sup>1,49</sup>

Randomized clinical trials in human and veterinary patients are lacking while a plethora of experimental and case series studies have been published in search of methods to attenuate local and distant effects of intestinal IRI. Table 1-1 includes additional interventions not covered in greater detail below.

### **Anti-oxidants**

Within the body naturally occurring anti-oxidants function to protect tissue from ROS formed during normal cellular processes.<sup>3</sup> Unfortunately, these anti-oxidants become overwhelmed during IRI and are unable to prevent deleterious consequences of ROS production.<sup>3,4</sup> Numerous animal studies have investigated ways to supply exogenous antioxidants and most studies have shown positive results.<sup>3,4,49</sup> However, the difficulty with many antioxidants is that their benefits are only seen if they are administered prior to ischemic insult; therefore, clinical use has been limited.

## **N-acetylcysteine (NAC)**

Glutathione is one of the most important endogenous antioxidants as it is able to prevent the formation of the hydroxyl radical by chelating iron and inhibiting lipid peroxidation.<sup>49</sup> It is produced primarily in the liver and cysteine is the rate-limiting amino acid in the formation of glutathione.<sup>49</sup> Administration of NAC has been studied as a method of enhancing the production of glutathione and NAC has decreased free radical damage in models of endotoxemia, radiation therapy, IRI, sepsis and ARDS.<sup>49,71,72</sup> N-acetylcysteine is available as a supplement, labeled as an antioxidant, and also as a pharmaceutical agent primarily used as a mucolytic and to treat acetaminophen toxicity.

## **Allopurinol**

Allopurinol prevents the formation of superoxide by competitively inhibiting XO.<sup>49</sup> Many studies have documented decreased IRI in many organs when allopurinol was administered prior to ischemia.<sup>49,73,74</sup> The clinical use of allopurinol is limited to organ transplantation.

## **Superoxide dismutase (SOD) and catalase**

Superoxide dismutase is an endogenous free radical scavenger that converts superoxide to less reactive hydrogen peroxide. Catalase then converts hydrogen peroxide to water.<sup>49</sup> Amyotrophic lateral sclerosis (Lou Gehrig's disease), is a genetic condition in which there is a defect in the SOD gene, resulting in accumulation of superoxide and subsequent neurological degeneration.<sup>49</sup>

SOD can be administered exogenously and significant benefits have been shown with its use in renal transplantation.<sup>75,76</sup> SOD is available over the counter as an antioxidant and anti-inflammatory supplement. Catalase can also be supplemented and

it is typically administered with SOD. Several animal studies document decreased IRI when catalase and SOD were administered prior to injury.<sup>77,78</sup>

## **Vitamins**

Vitamin E, or  $\alpha$ -tocopherol, is able to interrupt lipid peroxidation and scavenge ROS.<sup>49</sup> Vitamin C, or ascorbic acid, works in conjunction with vitamin E to block the chain reaction of lipid peroxidation by ROS and scavenge free radicals.<sup>49,79</sup> However, vitamin C is involved in the reduction of ferric to ferrous iron, a process that normally enhances iron absorption from the intestinal tract. During ischemia, the generation of ferrous iron is deleterious as it is required for the production of the hydroxyl radical.<sup>2,49</sup> Therefore, vitamin C has the potential for pro-oxidative effects. Nonetheless, studies have shown the administration of very high levels of vitamin C to be beneficial in certain conditions particularly when administered prior to injury.<sup>49,79</sup>

## **Deferoxamine**

This is an iron chelator that decreases available ferrous iron, thereby preventing or minimizing the production of the hydroxyl radical.<sup>49</sup> While iron chelation has shown benefit in models of IRI, there are potentially severe side effects of administration since iron is essential for oxygen delivery and many other biological processes.

## **Calcium channel blockers**

Calcium is involved in the conversion of XD to XO and the production of superoxide and other ROS. During IRI increased cytosolic calcium results from damage to cell membrane pumps. Administration of calcium channel blockers such as nimodipine and verapamil has been shown to improve blood flow in cases of “no-reflow phenomenon” and reduce ischemic injury.<sup>49</sup> Like other antioxidants, benefits are mostly seen when treatment is administered prior to ischemia.

## **Dimethyl sulfoxide (DMSO)**

Dimethyl sulfoxide is able to scavenge hydroxyl radicals.<sup>80</sup> Other purported benefits include vasodilation and platelet inhibition.<sup>80</sup> There are multiple studies investigating the use of DMSO in animal models of IRI showing mixed results.<sup>49,80,81</sup> DMSO interaction with tissue can also form the methyl radical that reacts with fat and/or oxygen to form free radicals. Therefore, it is possible that the amount of DMSO required to act as a scavenger of the hydroxyl radical may paradoxically result in cell damage.

## **Nitric oxide donors**

Therapies aimed at increasing NO, with anticipated vasodilation and improved hemodynamics have been studied and pretreatment with NO donors have shown benefit in small animal models of intestinal IRI.<sup>82,83</sup>

## **Ischemic pre-conditioning (IPC)**

To date, one of the most promising methods of minimizing IRI in the intestine and other organs is IPC.<sup>1,33</sup> This practice involves exposing an organ or tissue to brief ischemia prior to an extended duration of ischemia and subsequent reperfusion. The optimal amount of pre-ischemia time, number of pre-ischemia cycles, and time between pre and prolonged ischemia has not been confirmed.<sup>33</sup> The mechanism of IPC protection is believed to be mediated by adenosine, NO, heme-oxygenase, anti-apoptotic genes, decreased ROS production, and the generation of endogenous antioxidants.<sup>33</sup>

Animal models of intestinal IPC have shown decreased bacterial translocation, reduced expression of cell membrane adhesion molecules, and prevention of leukocyte rolling. The benefits of IPC can be exploited in cases of organ transplantation, however,

the vast majority of clinical veterinary intestinal IRI is unpredictable and therefore not amenable to IPC. To this end, Bretz et al. recently investigated the effects of ischemic *post*-conditioning in a rabbit model of intestinal IRI. In this study, rabbits were subjected to 45 minutes of intestinal ischemia, followed by 4 cycles of 30 seconds reperfusion/ 30 seconds re-occlusion and subsequent 2 hours of reperfusion. Unfortunately, no benefit of ischemic post conditioning was found in this model.<sup>33</sup>

## **Anti-inflammatory Therapy**

### **Anti-leukocyte**

Leukocyte activation can be inhibited by strategies that target TNF- $\alpha$ , PAF and leukotrienes.<sup>1,84</sup> Preventing the expression of leukocyte adhesion molecules on cell surfaces can mitigate the deleterious effects of leukocyte-mediated injury. Inhibitors of NF- $\kappa$ B have been found to decrease damage in models of IRI. Blocking leukocyte adhesion and rolling on endothelial cells can also attenuate leukocyte action.<sup>1,49,84</sup> The antioxidants allopurinol and SOD have shown benefit in this capacity. Anti-adhesion molecule antibodies are also being investigated.

### **Anti-complement**

Several experimental studies have found that complement inhibition through receptor antagonists or complement-deficient animals leads to significantly reduced intestinal IRI and secondary lung injury.<sup>1,13,14</sup>

## **Feeding and Supplementation**

### **Enteral feeding**

One of the most important and effective means of restoring intestinal mucosal integrity following injury is through enteral feeding. Studies in animals and humans have found that bacterial translocation and secondary organ injury are minimized when

feeding is instituted as soon as possible.<sup>1,85</sup> Furthermore, parenteral nutrition has been linked to increased leukocyte adhesion in the intestine and increased mortality compared to enteral feeding.<sup>86</sup>

### **Glutamine supplementation**

This amino acid is an important nutrient for intestinal epithelial cells and is considered critical in times of serious injury or illness. Supplementation either through enteral or parenteral means has been shown to decrease bacterial translocation and improve mucosal healing in models of intestinal injury.<sup>87,88</sup> The mechanism of glutamine's effects is by increasing glutathione and SOD production, decreasing TNF- $\alpha$  production, and reducing the expression of ICAM-1.<sup>87,88</sup>

### **Glycine supplementation**

Glycine is another amino acid that has been shown to provide anti-inflammatory and immunomodulatory effects in models of intestinal IRI.<sup>1</sup>

### **Improving Gastro-intestinal Perfusion and Oxygenation**

Shock, whether septic or otherwise, is a common cause of intestinal IRI in humans and animals. Aggressive resuscitation measures should be instituted with particular attention aimed toward improving blood pressure and splanchnic perfusion, beginning with intravenous crystalloid and colloid fluid delivery and administration of positive inotropes, if indicated. Furthermore, bacterial translocation can be minimized by providing supplemental oxygen using flow-by or intra-nasal cannulas and/or administering red blood cells. A study employing a hemorrhagic shock model in rats found that administration of 100% oxygen decreased bacterial translocation and TNF- $\alpha$  gene expression compared to animals that breathed room air.<sup>31</sup>

Another experimental method of improving intestinal oxygen delivery is through administration of perfluorocarbons (PFCs), molecules that can deliver 20-25 times more dissolved oxygen than plasma. These molecules also have a low-oxygen binding constant resulting in efficient release of oxygen in tissue. Intraluminal delivery of PFCs has been shown to preserve intestinal mucosal function and structure after IRI, and secondary lung injury was mitigated by use of peritoneal lavage using PFCs.<sup>1</sup> PFCs are considered a greenhouse gas with potential health hazards and are used in industrial settings as surfactants. Clinical use of PFCs has not been investigated.

### **Future Directions**

Many therapeutic approaches have been studied; however, no single treatment has been found to prevent the damage associated with IRI. It is likely that a multi-modal approach or novel technique will be necessary to successfully treat IRI.

Low-level laser therapy (LLLT) has been extensively investigated as a method of enhancing wound healing.<sup>89</sup> Photons emitted from lasers are absorbed by the mitochondria, resulting in increased cellular metabolism and ATP production. This is followed by an increased expression of growth factors, cytokines, and genes related to cellular proliferation and migration. Lasers enhance angiogenesis, increase collagen deposition, improve tensile strength, reduce the inflammatory phase of wound healing, and hasten wound closure in animal models.<sup>89</sup> Furthermore, laser therapy was demonstrated to decrease the production of reactive oxygen species by human neutrophils.<sup>90</sup>

The effects of laser in models of cardiac and skeletal muscle IRI have been studied.<sup>91-93</sup> It was found that laser irradiation of the heart in an isolated-perfused model resulted in significantly improved cardiac function, with increased anti-oxidant activity

and increased ATP stores compared to controls.<sup>91</sup> Others have shown a positive effect of laser therapy following epicardial irradiation in myocardial infarct models in rats and dogs.<sup>92</sup> Laser therapy was also shown to significantly increase anti-oxidants and decrease injury in skeletal muscle following IRI in a rat model.<sup>93</sup> Finally, percutaneous laser irradiation of the lungs following intestinal IRI was shown to decrease secondary lung injury in a rat model.<sup>94</sup>

Based on the demonstrated ability of laser to improve healing and the positive results of laser therapy in other models of IRI, we hypothesized that low level laser therapy applied directly to the injured intestine would decrease local and distant tissue injury in a rat model of intestinal IRI.

Table 1-1. Therapeutic strategies for treating intestinal IRI.

Intervention	Mechanism of action
N-acetylcysteine	Increase glutathione production
Glutathione	Anti-oxidant; exogenous supplementation has poor bioavailability
Allopurinol	XO inhibition; prevents ROS formation
SOD	Converts superoxide to hydrogen peroxide
Catalase	Converts hydrogen peroxide to water
Vitamin E, C	Inhibit lipid peroxidation; scavenge ROS
Deferoxamine	Iron chelation
Verapamil	Calcium channel blocker
DMSO	Scavenge hydroxyl radical
Albumin	Scavenges ROS
Mannitol	Scavenges ROS
21-aminosteroids	Scavenges ROS
NO, NO donors (FK-409)	Vasodilation, platelet aggregation inhibition, scavenges superoxide
Ischemic pre-conditioning	Increases endogenous production of antioxidants, Heme oxygenase-1, NO, adenosine, anti-apoptotic genes
Glutamine supplementation	Increase glutathione, SOD, decrease TNF-a and ICAM-1
Adenosine	Blocks neutrophil adherence and extravasation
Dexamethasone, corticosteroids	Inhibits phospholipase A2, decreased cell membrane release of phospholipids
Methyl prednisolone	Inhibits TNF and IL-6
CD11/CD18 monoclonal antibodies	Block neutrophil adhesion molecules
Pentoxifylline	Inhibits neutrophil function, increase glutathione
Ethanol	Inhibits neutrophil rolling, adhesion
LY-255283, SC-41930	Leukotriene B4 antagonists; prevent neutrophil chemotaxis, adherence
Elgin C, L658, 758	Elastase inhibitors; prevent neutrophil extravasation
Anti-neutrophil serum	Decrease number of circulating neutrophils
Hydroxyurea	Decrease number of circulating neutrophils
COX inhibitors	Decrease production of PG, thromboxanes
Soybean trypsin inhibitor, aprotinin	Protease inhibitors, decrease proteolytic effects of proteases
$\alpha$ -Melanocyte	NF-kB inhibition
Anti-TNF antibody	Inhibits TNF expression
IL-10	Decrease IL-6, TNF-a
CINC antibody, FR-167653	Inhibits TNF a, IL-1
Hepatocyte growth factor	Inhibits TNF
Hyperbaric oxygen	Inhibits TNF
L-Arginine	Inhibits TNF a, IL-6

Table 1-1. Continued

Intervention	Mechanism of action
Glycine	Inhibits neutrophil infiltration, IL-6
L-Propionyl carnitine	Inhibits neutrophil infiltration
Pirfenidone	Inhibits TNF $\alpha$
Rolipram	Inhibits TNF $\alpha$
WEB 2086, BN 52021, UK 74505	PAF antagonists; block PAF dependent neutrophil adhesion
Cyclic peptide AcF	Compliment antagonist
GPI 6150	Blocks P-selectin, ICAM-1
Heparin binding EGF	Decrease P, E selectin, VCAM-1, ICAM-1 expression
Lexipafant	PAF antagonist
M40401	Blocks P-selectin, ICAM-1
P-Selectin glycoprotein ligand	Blocks P-selectin
Volume resuscitation	Improve GI perfusion
Nasal oxygen	Improve GI perfusion, oxygen delivery
PFC	Improve GI oxygen delivery
Dopamine, dobutamine	B-agonist, improve perfusion, increase c-AMP, stabilize leukocytes, decrease MPO anti-inflammatory
Hypertonic saline (HTS)	Decrease PAF, leukocyte activation
Hetastarch	Decrease neutrophil function, prolong activity of HTS
Enteral feeding	Improve immune response to injury, increase IgA, mucin secretion, promote mucosal healing, decrease bacterial translocation
Therapeutic laser	Decrease secondary lung injury; decrease TNF $\alpha$ , IL-1, IL-6; increase NO, vasodilation; decrease neutrophil function/ MPO expression

## CHAPTER 2 THERAPEUTIC LASERS IN VETERINARY MEDICINE

### Introduction

Surgical lasers have been used in veterinary medicine for several decades; however, *therapeutic* lasers have only recently become widely available for use in animal patients.<sup>95</sup> Although recognition and acceptance of therapeutic lasers in veterinary medicine is in its infancy, the first scientific report of lasers used for a biomodulatory purpose was almost 50 years ago.<sup>96</sup> In 1967, a Hungarian scientist named Endre Mester performed an experiment using a low power, ruby (694 nm) laser in which he irradiated shaved areas on the backs of mice to see if the laser would cause cancer. Instead what he found was that the laser treated groups did not develop cancer, but their shaved hair grew back substantially faster than in the untreated group.<sup>96</sup> Over the subsequent decade, Mester and others described the ability of lasers to promote cutaneous wound healing and increase tissue collagen production, and observed that too little laser radiation would produce no effect and too much would be detrimental to healing.<sup>97-100</sup> Several other reports have since been published, both supporting and refuting the effects of laser therapy on tissue healing and pain modulation.<sup>95</sup>

This chapter will review basic laser physics and discuss the mechanisms behind “photobiomodulation” (modifying a biologic process by application of light).<sup>101</sup>

### Laser Physics

Lasers, the sun, ordinary light bulbs, x-ray machines, and microwave ovens, emit electromagnetic radiation.<sup>95,102</sup> Energy from these sources travels at the speed of light in packets known as photons. Photons travel in waves, not unlike sound waves, and

the type of radiation is distinguished by its wavelength. Electromagnetic radiation is a spectrum from very short (gamma rays, 1000-1 fm) to long (radio waves, 1000-1m) wavelengths. In the middle of this spectrum lies visible light (400-800 nm) and invisible infrared (1000-0.8  $\mu\text{m}$ ), and laser radiation falls within these wavelengths.<sup>95,102</sup>

Laser is an acronym for “light amplification by stimulated emission of radiation”.<sup>95,102</sup> In this process, electromagnetic energy is harnessed into an intense, coherent, monochromatic beam of light. The properties of monochromaticity (all waves are the same length and color if within the visible spectrum) and coherence (all waves are in phase) are characteristics of all lasers. This is in contrast to light emitting diodes (LEDs) which do not emit coherent light.<sup>95,103,104</sup> The biomodulatory effects of lasers are believed to be due, in part, to their coherence; however, there is some limited evidence to suggest that LEDs can be effective in photobiostimulation.<sup>103-105</sup>

A laser beam is emitted when a material (gas, liquid, or solid) is stimulated by an external energy source, such as electricity, to release photons of a single color or wavelength.<sup>95</sup> For example, a helium-neon (HeNe) laser will emit only wavelengths of approximately 633 nm, which fall within the visible light spectrum and produce the color red. Gallium arsenide (GaAs) lasers emit wavelengths of 904 nm, and are thus invisible within the infrared portion of the spectrum.<sup>89</sup>

In addition to wavelength, other parameters are used to characterize lasers.<sup>89,106</sup> The power of a laser is measured in watts (W) or milliwatts (mW) and describes the *rate* at which energy is used.<sup>106</sup> This should not be confused with the *amount* of energy delivered, which is measured in Joules (J), and  $1\text{ W} = 1\text{ J/second}$ .<sup>106</sup> Power output is significant, because a laser with a higher wattage will deliver the desired energy (or

dose) more quickly but have a greater capacity to heat or burn tissue, particularly dark pigmented skin.<sup>106,107</sup> Power output can be measured using a power meter, and some commercial lasers will have a meter provided within the device to allow the user to regularly check that the output is within the intended range. It is important to check power output on a regular basis as a means of quality control and assurance that the intended dose is delivered.

Therapeutic lasers are commonly referred to as “low level lasers” or “cold lasers”, in contrast to high-powered lasers that are used to cut or ablate tissue. Low level lasers have a power output less than 500 mW and cannot cut tissue.<sup>95</sup> Amongst low level lasers, power output (mW) varies greatly, anywhere from 5 mW to 500 mW.<sup>107</sup> Recently, lasers with power output greater than 500 mW (up to 16 W) have been marketed as therapeutic lasers. These lasers are not considered “low-level” or “cold lasers” because they have the capacity to cause considerable tissue heating and skin burning, but they may still provide therapeutic benefits as are seen with lower power lasers.<sup>95,107</sup>

Lasers are divided into four classes with additional subclasses: 1, 2, 3a, 3b, 4. A common misconception is that these classes distinguish the efficacy or quality of the laser. Rather, laser class designates the hazards associated with laser use, in particular the ability to cause eye injury, and is based on power output, parallelism, diameter of beam, exposure time, and wavelength.<sup>95</sup> Class 1-3a lasers, including supermarket scanners, laser pointers and remote controls, are considered safe. Class 3b lasers pose a risk of retinal injury by direct illumination, and eye protection is recommended. Class 4 lasers are considered to be an acute hazard to the skin and

eyes from direct and scattered radiation and eye protection is mandatory. Class 4 lasers are only available for purchase by medical professionals.<sup>95</sup>

Laser energy can be delivered in either a continuous or pulsed manner.<sup>108</sup> When a laser is pulsed, the power output will reach a peak and return to zero at varying frequencies (hertz, Hz) and duty cycles. Therefore, the amount of power delivered will be the average power output. While most research has been conducted using continuous wave (CW) laser, there is recent speculation that pulsed delivery may have beneficial effects.<sup>108</sup> From a physical standpoint, pulsing delivery causes less heat accumulation within tissue but requires a longer treatment time to deliver the same desired dose compared with CW. The difference in treatment time would depend on the average power and pulse frequency. The optimal pulse frequency has not been determined.<sup>108</sup> Values ranging from 2.5-20,000 Hz are beneficial in various models of wound healing and pulse frequencies between 4- 8,000 Hz have demonstrated efficacy for pain relief.<sup>108</sup>

Power density, also known as intensity or irradiance, is the amount of power concentrated in a given area and is measured in  $W/cm^2$ .<sup>106,109</sup> If the laser beam is spread over a larger area (larger spot size), the amount of energy at each point becomes less, compared to concentrating the energy at a single, small point. This will be influenced by the beam diameter (millimeters) and spot size ( $cm^2$ ) that are specific to each laser. Some lasers allow for adjustment of beam diameter and spot size. Many scientists, including Mester, have recognized the importance of irradiance on cellular response.<sup>100,109</sup> Various studies have shown that an optimal irradiance exists (model-

dependent), whereas energy densities above and below this value result in either no or adverse tissue response.<sup>100,109-112</sup>

Dosage (also known as energy density or fluence) appears to be the most important laser parameter in clinical application.<sup>5,106,113-116</sup> Dosage is measured in J/cm<sup>2</sup>, and can be calculated using the following formula:

$$\text{Dose} = \frac{P \times t}{A}$$

P= laser's output power (W)

t= treatment time (seconds)

A= area treated (cm<sup>2</sup>)

The usefulness of this formula is evident. By adjusting treatment time in relationship to a laser's output power, one can insure that the intended dosage is delivered. Lasers that are available commercially and marketed for medical or veterinary use will likely have recommended doses for various conditions pre-programmed into the unit or listed in the instruction manual. While the optimal treatment dose has not been established for any condition, generally recommended doses fall between 2-10 J/cm<sup>2</sup>.<sup>5,116</sup>

One last consideration is the depth of laser penetration. This depends on the wavelength of the laser and the interaction of that wavelength with water, melanin and hemoglobin within tissue.<sup>106,109</sup> An optical window exists between approximate wavelengths of 800-905 nm where the least amount of energy is absorbed by water and pigment, resulting in the most amount of energy transmitted through superficial tissue and absorbed by the tissue of interest.<sup>109</sup> Therefore, if the target tissue is superficial, wavelengths in the 630 nm spectrum will be sufficient whereas longer wavelengths are

needed to reach deeper tissue. A GaAs laser (904 nm) can reach tissue depths of 3-5 cm, while a HeNe (633 nm) will have more superficial penetration, near 1cm.<sup>95,108</sup>

Besides the wavelength of the laser in use, other factors may influence the depth of penetration, including hair coat, skin coloration, and tissue composition. Much of the radiation energy may be absorbed by hair, dark pigmented skin, and highly vascular tissue such as muscle.<sup>95,108,109</sup> Reflection of light from the tissue surface can decrease absorption. Therefore, when targeting tissue deep to the skin, the laser should be held in contact with the skin to reduce reflection (providing that the power is low enough not to cause tissue heating/ burning).

## **Photobiomodulation**

### **Tissue Healing**

The effects of surgical or “hot” lasers that instantaneously heat and cut tissues are well known. However, because many therapeutic lasers do not cause discernible heating and do not cause immediate macroscopic tissue changes, skepticism about their effect is understandable. However, there are numerous *in vitro* and *in vivo* studies documenting the effects of low level lasers.<sup>5,89,114,117-123</sup> These effects are initially seen at the cellular and sub-cellular level, but can manifest as perceptible changes in pain response and tissue healing.<sup>5,89,123</sup>

The interaction of lasers and mammalian tissue can be likened to the process of photosynthesis in plants, where light is absorbed by chlorophyll (a chromophore) and a chemical reaction takes place that leads to the production of cellular byproducts. In animals, laser energy is absorbed by the chromophore cytochrome c oxidase in the mitochondria.<sup>5,105,109,124</sup> This results in photodissociation of nitric oxide (NO) from cytochrome c oxidase, thereby allowing oxygen to bind in its place and promote

mitochondrial respiration.<sup>120</sup> This then increases cellular metabolism and adenosine triphosphate (ATP) production and stimulation of deoxyribonucleic acid (DNA) formation.<sup>109,113</sup> These events are followed by an increased expression of growth factors, cytokines, and genes related to cell proliferation and migration.<sup>109,119,120,125</sup>

Laser therapy can affect each stage of tissue healing (inflammation, proliferation, and maturation).<sup>89</sup> The role of laser in the inflammatory phase is that of immunomodulatory—some studies have shown enhancement of the inflammatory response and increased production of growth factors such as transforming growth factor beta one (TGF- $\beta$ 1), while others show a reduction of inflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ) and prostaglandin E2 (PGE-2) and inhibition of cyclo-oxygenase 2 (COX-2).<sup>114,119,121,125-133</sup> Yu et al. demonstrated the ability of laser therapy to enhance the immune response during acute septic inflammation. This group of investigators created a septic peritonitis model and found that rats irradiated with laser had an increased number of lymphocytes, increased mitogenic response of lymphocytes, and an overall increased survival rate, compared to non-irradiated rats.<sup>128</sup> Others have demonstrated acceleration of the inflammatory phase, with rapid progression into the proliferative phase of healing.<sup>114,121,133</sup> Furthermore, laser therapy has been shown to reduce inflammatory mediators, such as COX-2 and PGE-2, and inhibit the activity of inflammatory cells and development of edema.<sup>118,125</sup>

An increase in vascular activation (hyperemia) has been seen within the first 36 hours following laser treatment of open wounds.<sup>133</sup> This may be due to a local, sub-sensory increase in temperature, which can influence cell membranes and ion exchange and, ultimately, vascular tone. Laser therapy of rat intestinal microcirculation

led to a decrease in vascular smooth muscle cytosolic calcium concentration, resulting in potent arteriole dilation of the irradiated vessel and increased blood flow.<sup>134</sup> Others have found that irradiation of open cutaneous wounds results in increased granulation tissue and neovascularization, within 3 days following wound creation and initiation of laser treatment.<sup>114,121</sup>

While laser therapy does appear to influence the inflammatory phase, it is clear from a large number of studies that the proliferative phase of wound healing is greatly enhanced by low-level lasers.<sup>5,89,117,118,120,122,126-128,135-137</sup> Work done using cell cultures has found that various wavelengths and doses are effective at increasing fibroblast proliferation, but HeNe laser (633 nm) at a dose of 5 J/cm<sup>2</sup> appeared to stimulate the greatest amount of cell proliferation and migration.<sup>113</sup>

Similar findings have been demonstrated in animal models of wound healing. Medrado et al. created skin wounds on the dorsum of rats and applied various doses of laser therapy.<sup>118</sup> This team found that at an optimal dose of 4 J/cm<sup>2</sup>, there was significantly greater proliferation of myofibroblast and collagen deposition in treated wounds, compared to controls. Another group reported that laser treatment of sutured wounds resulted in increased tensile strength at 7 days post operatively compared to non-irradiated wounds.<sup>122</sup> Histologic specimens from this experiment showed significantly greater collagen deposition in the gap of irradiated wounds.<sup>122</sup> Furthermore, it has been shown that laser therapy can stimulate epithelialization and collagen deposition in animal models of diabetes and glucocorticoid excess.<sup>132,137-139</sup>

The final phase of wound healing is maturation or remodeling of scar tissue. Lasers influence this phase by enhancing the organization of collagen fibers within

wounds.<sup>118,135,140</sup> To this end, improved healing of tendons and ligaments has been reported.<sup>135,140</sup> Furthermore, there are reports of using laser therapy (particularly 585 nm) to treat hypertrophic scars in humans.<sup>141,142</sup>

### **Ischemia Reperfusion Injury**

There is evidence to suggest that lasers can diminish local effects of ischemia/reperfusion injury in skeletal and cardiac muscle and mitigate secondary lung injury following intestinal ischemia.<sup>6,91-94,143-145</sup> Laser irradiation applied following experimental occlusion of the blood supply to the gastrocnemius muscle of rats resulted in significantly decreased muscle injury compared to non-irradiated controls.<sup>6,93</sup> The mechanism of this action was reportedly due to induction of antioxidants and increased levels of the cytoprotective protein heat shock protein-70i.<sup>93</sup>

Canine and rat models of chronic myocardial infarction have also shown positive effects related to laser treatment.<sup>91,92,143-145</sup> Following epicardial irradiation, the infarct size and mortality rate were significantly lower for treated animals compared to controls. These results correlated with a reduction in mitochondrial damage and an increase in ATP levels.<sup>92,143</sup> Increased ATP levels and antioxidant activity were additionally demonstrated following laser therapy in an *ex vivo* heart transplant model.<sup>91</sup> Finally, Tuby et al. found upregulation of vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) expression in infarcted rat hearts following laser therapy, which was linked to increased angiogenesis and cardioprotection.<sup>145</sup>

De Lima et al. investigated the specific effects of laser on pulmonary injury following intestinal IRI.<sup>94</sup> In that study, rats underwent 45 minutes of superior mesenteric artery occlusion followed by percutaneous low level laser irradiation of the right upper bronchus. Rats were euthanized after 4 hours of reperfusion. This study concluded

that laser treatment following intestinal IRI resulted in decreased lung edema and inflammation mediated through decreased myeloperoxidase activity and TNF- $\alpha$  generation within the lung parenchyma.<sup>94</sup>

## **Pain Relief**

Not long after Mester demonstrated the ability of lasers to modulate tissue healing, Friedrich Plog showed that lasers could provide an alternative to needle acupuncture for pain relief.<sup>146</sup> Since then lasers have been used to effectively manage pain from various causes, including osteoarthritis, tendonitis, mucositis, muscle injury, plantar fasciitis and back pain.<sup>116,123,147-153</sup> The United States Food and Drug Administration has approved laser therapy for treatment of head and neck pain and carpal tunnel syndrome.<sup>147</sup>

The mechanism by which laser decreases pain is not as well understood as its effects on tissue healing. This is partly due to the complexity of pain pathways and difficulty in measuring pain in experimental studies. Nevertheless, several studies have begun to explain the processes involved in photobiomodulation of pain.<sup>147,148</sup> One of the primary means of decreasing pain (either pharmacologically or otherwise) is by decreasing the production of inflammatory mediators. Numerous studies have shown the ability of laser to decrease the production of PGE-2 and TNF- $\alpha$ , and inhibit COX-2.<sup>129,130,147,154</sup> Others have compared laser treatment to traditional non-steroidal anti-inflammatory treatment for rheumatoid and gout arthritis, and found laser to be more effective than pharmacologic intervention in treating these chronic pain conditions.<sup>149,155</sup>

Besides decreasing inflammation, laser therapy is believed to reduce pain through several other mechanisms that are particularly important when treating chronic pain. Photochemical reactions take place at a subcellular level that lead to increased ATP

and stabilization of cell membranes and ATP-mediated pumps.<sup>5,105,109,124</sup> This in turn results in restoration of the cell membrane electrochemical gradient and resting membrane potential. Excessive or inappropriate depolarization of peripheral nociceptors is minimized and the conduction of pain along A $\delta$  and C fibers is slowed.<sup>147,150,156</sup> Other postulated mechanisms of pain relief include increased release of B-endorphins and serotonin, and enhanced removal of inflammatory mediators from the site of injury due to laser-induced changes in local hemodynamics.<sup>147,150,157,158</sup>

### **Clinical Application**

The biomodulatory effects of laser therapy in wound healing include vasodilation, angiogenesis, increased collagen synthesis by fibroblast, differentiation of fibroblast into myofibroblasts, stimulation of leukocytes, and enhanced antioxidant activity.<sup>6,91-94,114,117,118,120,121,122,128,135,143-45</sup> The end results of these processes are improved tissue healing and regeneration, increased wound contraction, increased strength of repaired tissue, improved immune function, and defense against ischemia reperfusion injury.<sup>5,89,94</sup> Laser therapy can also reduce pain through several mechanisms, including reduction of inflammation, modulation of pain transmission and stimulation of endogenous opiate release.<sup>147,149</sup>

Based on the aforementioned properties of laser, the current indications for use in human and veterinary medicine are numerous, but primarily include enhancement of cutaneous wound and tissue healing and amelioration of acute and chronic pain.<sup>5,147-149,159-161</sup> Because laser is recognized to enhance neovascularization, irradiation of tumors or wounds that may contain cancer cells is contraindicated.<sup>95,114</sup> Lasers pose a known risk to the eye, so irradiation of or near the eye should not be performed.<sup>95</sup>

Additional contraindications include irradiation over a pregnant uterus and open growth plates.<sup>95,135</sup>

A therapeutic window for photobiostimulation is an important concept.<sup>5,98,100,109,113-116</sup> At sub-therapeutic doses, cells will not be stimulated and no reactions will occur; at extremely high doses, detrimental effects can be seen. The modulatory effects may also be wavelength specific and vary with other laser parameters such as power density, collimation or pulse frequency.

Table 2-1. Comparison of three therapeutic lasers with different wavelengths and power output.

	Helium Neon (HeNe)	Galium Arsenide (GaAs)	Galium Aluminum Arsenide (Ga Al As)
Wavelength (nm)	650	904	970
Power output	70 mW	500 mW	10 W
Pulsed	Continuous or pulsed	Pulsed	Continuous or pulsed
Class	3b	3b	4
Primary use	Superficial wound healing, research	Pain relief, wound healing	Deep tissue pain relief and tissue healing

## CHAPTER 3 THE EFFECTS OF LOW LEVEL LASER THERAPY (LLLT) ON A RAT MODEL OF INTESTINAL IRI

### **Introduction**

Ischemia-reperfusion injury (IRI) is a complex cascade of events beginning with the depletion of oxygen to cells, exhaustion of adenosine tri-phosphate (ATP) stores, impairment of cellular membrane function, and accumulation of toxic metabolites within cells.<sup>1-4</sup> When perfusion is restored, reactive oxygen species are quickly generated which overwhelm endogenous anti-oxidant mechanisms, leading to further cellular dysfunction. The deleterious effects of IRI can be demonstrated both in local and distant tissue.<sup>1-4</sup>

The gastrointestinal tract is particularly sensitive to IRI and gastrointestinal ischemic injury is a common cause of morbidity and mortality in human and veterinary patients.<sup>1-4</sup> Treatment of ischemic conditions typically requires surgical resection of any potentially damaged bowel. However, in some cases the portion of bowel that is affected is extensive or located in an area that is not amenable to resection. Furthermore, even if surgical resection is successful, the consequences of reperfusion injury can be seen in distant organs. Many therapeutic approaches to treating IRI have been studied; however, no single treatment has been found to prevent the damage associated with IRI.<sup>41,49</sup>

Based on the demonstrated ability of LLLT to improve healing and the positive results of laser therapy in other models of IRI, we hypothesized that LLLT therapy would decrease local and distant tissue injury in a rat model of intestinal IRI.

Null Hypothesis: LLLT at the parameters tested does not protect against intestinal ischemia-reperfusion injury in the rat in the acute (0-6 hour) phase.

Alternative Hypothesis: LLLT at the parameters tested protects against intestinal ischemia-reperfusion injury in the rat in the acute (0-6 hour) phase.

The objectives of this study were to validate a rat intestinal IRI model and investigate the short-term effects of LLLT applied to the intestinal serosal surface following IRI.

## **Materials and Methods**

### **Animals**

This study was approved by the University of Florida Institutional Animal Care and Use Committee and was performed in accordance with the Institute for Lab Animal Research Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 300-400 g were maintained in a temperature controlled room with alternating 12:12-hour light-dark cycles in an animal facility at the University of Florida. Animals were fed a standard diet and allowed free access to water.

### **Treatment Groups**

Ninety-six rats were randomly assigned to one of the following 15 treatment groups (Table 3-1): Laser treatment pre-reperfusion (TPr) with 0, 1 or 6 hours reperfusion; laser treatment post-reperfusion (TPo) with 0, 1, or 6 hours reperfusion; Control 1 (C1)--ischemia and reperfusion, no laser, with 0, 1 or 6 hours reperfusion; Control 2 (C2)—laser treatment, no ischemia and reperfusion, with 0, 1 or 6 hours of “reperfusion” time; Sham—anesthesia, vessel isolation, no ischemia and reperfusion and no laser with 0, 1 or 6 hours “reperfusion.” Ischemia time was 60 minutes and animals in Sham and C2 groups were under anesthesia for this time period without vessel clamping. Reperfusion time of 0 indicates the end of ischemia and beginning of

reperfusion. The reperfusion time started when the vascular clamp was removed or 60 minute duration of sham ischemia was complete.

### **Anesthesia and Monitoring**

Animals were placed in an induction chamber and anesthetized with isoflurane vaporized in oxygen. Anesthesia was maintained with 1-3% isoflurane vaporizer setting and 100% oxygen, initially by facemask. A tracheostomy tube (16 g catheter) was placed to secure an airway, to maintain anesthesia, and provide assisted ventilation. Rats were placed on a circulating warm water blanket and rectal temperature was maintained at  $37^{\circ} \pm 1^{\circ}\text{C}$  by altering the temperature of the heating blanket. The femoral artery and vein were catheterized using polyethylene tubing (Polyethylene-10, Intramedic, Clay Adams, Parsippany, NJ). The arterial catheter was used to monitor continuous direct blood pressure and the venous catheter used to administer crystalloid fluids to maintain mean arterial blood pressure at  $90 \pm 10$  millimeters of mercury (mmHg). Pulse oximetry and a Doppler probe were used to monitor oxygen saturation and heart rate, respectively. The pulse oximeter probe was placed over a hind paw and the Doppler probe directly over the heart.

### **Laser Parameters**

A diode laser with wavelength 650 nm (Healing Lasers Design Company, Cape Girardeau, MO, model HDC1 REV B) was used. The output power was measured using a power meter (Newport Corporation Field Master, Irvine, CA) and determined to be 70 milliwatts (mW) with continuous (non-pulsed) output at 1 cm from the tip. Based on pilot study data, the length of jejunum was 80-90 cm and the height of the intestine was 0.5 cm. To standardize beam diameter, a 1-cm standoff was constructed and applied to the treatment end of the laser probe with a  $1\text{cm}^2$  footprint. A dose of 0.5

Joules (J)/cm<sup>2</sup> was delivered using a point-to-point method whereby the probe was held over a 1cm length x 0.5 cm height (area, A= 0.5 cm<sup>2</sup>, Power density 35 mW/cm<sup>2</sup>) section of bowel for 3.5 seconds, then moved over to the next point immediately adjacent. This was repeated for the length of intestine on one side (between mesenteric and anti-mesenteric borders), and then the jejunum was turned over so that the opposite side (180 degrees from first side) was treated in the same manner. The total treatment time was 600 seconds (10 minutes).

### **Procedures**

After tracheal intubation and vascular catheterizations were completed, a ventral midline celiotomy was performed. A circumferential 5 mm jejunal biopsy was taken approximately 10 cm orad from the cecum. The ends of the biopsy site were ligated using 3-0 Polyglyconate (Maxon, Sherwood, Davis & Geck, St. Louis, MO). Based on results from a pilot study, this area was predictably the most grossly affected following ischemia and reperfusion injury. These biopsy samples were used to establish that no injury was present prior to intervention and for comparison between groups prior to intervention.

The superior mesenteric artery (SMA) was isolated. In treatment (TPr, TPo) and C1 groups, the SMA was occluded using two atraumatic vascular clamps (Accurate Surgical and Scientific Instruments, Westbury, NY) for 60 minutes. During ischemia, the small intestines remained in situ and the abdomen temporarily closed using towel clamps. Following ischemia, the vascular clamps were removed and the abdomen closed using 3-0 Polyglyconate.

Animals in group TPr received laser treatment for the last 10 minutes of ischemia, before the vascular clamps were removed. Laser treatment was applied for 10 minutes

immediately following vascular clamp removal in the TPo group. In the Control 2 group (rats not undergoing ischemia) laser treatment corresponded to the last 5 minutes of ischemia and the first 5 minutes of reperfusion in the ischemia groups.

During laser treatment, the jejunum and cecum were exteriorized from the abdomen and placed through a fenestration in a surgical drape. The laser was held with the 1-cm stand-off in contact with the serosal surface of the jejunum. Treatment was delivered to both sides of the intestine beginning at the cecum and working orad to the duodenum, so that the entire length of jejunum was treated equally. Sterile saline was used to keep the intestines moist during laser treatment.

Animals were maintained under anesthesia until the designated reperfusion time was reached, unless physiologic parameters could not be maintained within normal reference ranges and death appeared imminent. Immediately before euthanasia, 1 mL of blood was obtained from the caudal vena cava, a median sternotomy was performed and the left caudal lung lobe removed. Rats were then immediately euthanized by intracardiac injection of pentobarbital sodium (Euthasol, Diamond Animal Health, Des Moines, IA). The entire length of small intestine and a sample of liver were collected after euthanasia. Samples of lung, liver and intestine were immediately placed in 10% formalin for histologic analysis. The blood sample was immediately spun in a centrifuge, and serum was collected and stored along with samples of intestine, lung and liver at -80 °C for subsequent analysis.

### **Histologic Examination**

Five-millimeter transverse sections of the intestine were taken 20 cm from the duodenum and 20 from the cecum. Pilot Study results indicated that the area 20 cm from the duodenum represented the least severely affected area of jejunum and the

most severely affected area was 20 cm from the cecum. Transverse sections of lung and liver were taken at the mid-portion of the lobes. Tissues were processed using hematoxylin and eosin staining. Histologic grading was performed by a veterinary pathologist blinded to treatment groups. Intestinal injury was scored based on a previously described scale (Table 2).<sup>23</sup> Lung and liver tissue were evaluated for hemorrhage and edema, with scores of 0=normal (no hemorrhage or edema), 1=minimal, 2=mild, 3=moderate, and 4=severe.

### **Statistical Analysis**

Statistical calculations were performed using a computer software program (SAS PROC MIXED, SAS 9.1, Cary, NC).<sup>162</sup> The design was a three-factor analysis of variance with the fixed factors of laser dose (0, 1), ischemia (yes or no), and sacrifice time (0, 1, 6). The response variables were: intestinal injury at 3 locations: orad, aborad and biopsy; lung edema, and lung hemorrhage. Laser treatment pre and post reperfusion were compared by means of an unpaired t test and the Wilcoxon rank sum test. A p value <0.05 was considered significant.

## **Results**

### **Model**

The first objective of this study was to validate a model of intestinal IRI in the rat. Animals in the sham group did not develop intestinal injury after 6 hours of anesthesia ( $p= 1.0$ ) indicating that an abdominal incision, intestinal manipulation, and anesthesia did not contribute to intestinal injury. There was a significant difference in intestinal injury in rats that had IRI and no laser treatment (C1) compared to sham surgery, and the degree of injury was worse following 1 and 6 hours of reperfusion ( $p<0.0001$ ) (Figure 3-1).

No animals in the sham or C2 (no IRI, yes laser) groups were euthanized before the completion of reperfusion time. In the C1, 6 hour group, 4 of 7 animals were euthanized before the full 6 hours of reperfusion was reached. In the TPr and TPo groups, 2 of 12 animals were euthanized prior to the 6 hour designated reperfusion time. This difference was not significant ( $p=0.13$ ). The animals that were euthanized prior to the end of the reperfusion time were sacrificed based on failure to maintain hemodynamic parameters as measured using arterial blood pressure, pulse oximetry, and Doppler. Animals that were euthanized developed severe hypotension and hypoxemia and euthanasia was performed once it became clearly evident that death was imminent, per IACUC protocol. These changes only occurred in animals that underwent ischemia and greater than 1 hour of reperfusion, suggesting that the model (anesthesia) was not responsible for these changes in status.

### **Laser Treatment**

There were no significant differences between laser treatment pre (TPr) or post (TPo) IRI in any response variables; therefore, all treatment groups (Tx) were pooled for statistical analysis. Intestinal injury was significantly worse ( $p<0.0001$ ) in animals treated with laser and no IRI (C2) compared to sham (Figures 3-1, 3-2). Intestinal injury was significantly worse in Tx animals at all time points compared to sham ( $p<0.001$ ) (Figure 3-2). In animals that underwent IRI, those treated with laser (Tx) had significantly worse intestinal injury compared to those that did not have laser (C1) at 0 ( $p=0.0104$ ) and 1 ( $p=0.0015$ ) hour of reperfusion. After 6 hours of reperfusion there was no significant difference in injury between these two groups (Figure 3-2).

## Secondary Organ Injury

There were no significant lesions in any liver specimens. Lung injury (edema and hemorrhage) was not significant in sham animals (surgery and anesthesia without ischemia or laser did not contribute to lung injury). Lung injury was worse in animals that underwent ischemia and reperfusion compared to those without injury (Sham, C2). Lung injury was significantly worse after 1 hour of reperfusion compared to 0h ( $p=0.0089$ ), that is, lung injury was worse following ischemia *and* reperfusion compared to ischemia alone. There was no difference in lung injury between 1 and 6 hours. There was significantly less lung injury following IRI in treated animals (Tx) compared to untreated animals (C1) ( $p<0.001$ ) indicating a protective effect of laser treatment on the lung (Figures 3-3 and 3-4).

## Discussion

Intestinal IRI is associated with high morbidity and mortality in humans and animals.<sup>1,2</sup> Numerous techniques have been investigated in an attempt to minimize or prevent the deleterious effects of ischemia and reperfusion.<sup>1,3,33,49</sup> This rat model investigated a novel treatment method employing LLLT applied directly to the injured tissue. There were two primary findings of this study: 1) the dose and parameters of laser used in this model did not protect the intestine from acute IRI, and 2) LLLT of intestine damaged by IRI prevented secondary lung injury.

After 60 minutes of small intestinal ischemia, a dose of  $0.5 \text{ J/cm}^2$  LLLT did not prevent immediate intestinal damage. There was no significant difference in histologic damage when LLLT was performed before or after the start of reperfusion. Intestinal injury became more severe as reperfusion time increased up to 6 hours. After 0 and 1 hour of reperfusion, LLLT groups had significantly worse intestinal damage compared to

untreated animals. After 6 hours of reperfusion, this difference was no longer significant; however, injury was severe with histologic evidence of transmural damage in both treated and untreated groups (Figure 3-1). There are several explanations for these results including the laser parameters chosen for study and limitations inherent to the model.

Low level laser therapy has been widely investigated as a method of enhancing tissue healing, decreasing inflammation, and providing pain relief.<sup>89,120,130,147</sup> Furthermore, LLLT has also been shown to decrease ischemia and reperfusion injury in cardiac and skeletal muscle.<sup>91-93</sup> Although there are numerous studies that demonstrate the beneficial effects of LLLT on injured or inflamed tissue, there are other studies that show either detrimental or no effects.<sup>113,163-165</sup> The greatest challenge in designing a LLLT study is choosing irradiation parameters.<sup>106</sup> The parameters that can be altered include wavelength (nm), power (W), irradiance ( $W/cm^2$ ), fluence or dose ( $J/cm^2$ ), pulse structure (continuous wave vs. pulse frequency in Hz), beam diameter (mm), and dosing schedule (timing and frequency of treatment). Altering any one of these parameters could cause different biological effects and optimal treatment parameters have not been identified.<sup>106,110,111,113,</sup>

Laser therapy has been shown to follow the principles of the “Arndt-Schultz Law”, which states that a therapeutic window exists in which optimal treatment effects will be gained; doses outside this window will have minimal to no effect or could have detrimental effects.<sup>166,167</sup> In this regard, lasers are similar to many other treatments that are known to have biostimulatory effects at low doses and inhibitory effects at higher doses.<sup>168</sup> For example, Hawkins et al. investigated the effects of a helium-neon laser

on the proliferation of wounded fibroblasts in cell culture and found that a dose of 5 J/cm<sup>2</sup> enhanced cell viability and proliferation.<sup>113</sup> Increasing the dose to 10-16 J/cm<sup>2</sup> decreased cell proliferation and viability, and caused cellular and deoxyribonucleic acid (DNA) damage.<sup>113</sup> Others have found a biphasic response that correlates to irradiance (mW/cm<sup>2</sup>).<sup>101</sup>

The laser dose used in this study was 0.5 J/cm<sup>2</sup>, which is lower than the World Association of Laser Therapy (WALT) guidelines for treating human cutaneous tissue. WALT provides suggested doses for treating musculoskeletal conditions in humans, with the lowest recommended dose being 1 J/cm<sup>2</sup> or 1 J/point for treating the finger joints with a 904 nm laser. Our dose was lower than the recommended WALT dose because we took into account that our treatment was directly applied to the intestine (with a stand off) and no other tissue or pigments were between the laser diode and the treated tissue; therefore, a greater amount of laser energy was delivered to the target tissue rather than a portion potentially being attenuated or absorbed by melanin and, to a lesser degree, hemoglobin in overlying tissue.

This experiment was initially designed to investigate several different laser doses: 0.5, 1 and 5 J/cm<sup>2</sup> based on previous data suggesting these as optimal doses in various experimental and clinical scenarios.<sup>91-93,169,170</sup> However, the histological results that we found indicating that our dose actually caused *increased* local tissue damage compared to sham groups suggests that perhaps higher irradiance would *not* be beneficial. Yet, this dose provided protection against secondary lung injury, indicating that the dose was not too low to have a physiological effect. Nonetheless, it is likely that the parameters chosen were outside of the therapeutic window.

It is possible that the dose was not the source of injury but that the power density or irradiance ( $35 \text{ mW/cm}^2$ ) was inappropriate. There are numbers of studies that show benefits with irradiances above (up to  $100 \text{ mW/cm}^2$ , as recommended by WALT as the maximum irradiance) and below the irradiance we used.<sup>92,171-176</sup> For example, Oron et al. showed that an irradiance of  $5 \text{ mW/cm}^2$  reduced heart infarct size compared to  $2.5$  and  $25 \text{ mW/cm}^2$ .<sup>92</sup> Others have also found that there is an optimal irradiance and fluence that when coupled, provide benefit over other combinations of each variable.<sup>109,110</sup> Therefore, we can only conclude from this study that either or both the irradiance and/or fluence used were not optimal and other treatment parameters should be investigated. Future studies would be required to test this theory.

Another potential limitation of the study was the method of laser delivery. The treatment was delivered in a point to point manner but the laser beam size was such that the entire surface of the bowel was being treated—both mesenteric and anti-mesenteric surfaces. Histological examination of the tissue did not show a significant difference between mesenteric and anti-mesenteric surfaces with regard to degree of injury. Care was taken during delivery of the therapy to ensure as best as possible that every part of the jejunum was treated. However, human error is such that there were certainly millimeters of tissue that may have been missed. Nonetheless, this would be the case in a clinical treatment protocol. Furthermore, studies have shown that the effects of laser are not confined to the tissue that is directly treated.<sup>101</sup> Increased expression of cytokines and growth factors in the area treated are able to have a paracrine effect on surrounding tissue.

Although this study found a detrimental effect of LLLT on the intestine, an important finding was that secondary organ injury to the lungs was significantly decreased. The lungs are particularly sensitive to injury following primary organ IRI.<sup>9-12</sup> Intestinal IRI has been shown to increase pulmonary microvascular permeability and neutrophil infiltration.<sup>9</sup> These events are mediated by pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-  $\alpha$ ), increased expression of leukocyte integrins, and upregulation of pulmonary endothelial membrane proteins such as intracellular adhesion molecule one (ICAM-1).<sup>10,11</sup>

Our results found significantly less pulmonary edema and hemorrhage in animals treated with LLLT. Low level laser therapy decreased the expression of inflammatory mediators in other studies. For example, Safavi et al. found that laser irradiation of rat gingival mucosa led to decreased gene expression of interleukin one (IL-1) and interferon gamma (IFN- $\gamma$ ).<sup>170</sup> De Lima et al. recently investigated the specific effects of LLLT on pulmonary injury following intestinal IRI.<sup>94</sup> In that study, rats underwent 45 minutes of superior mesenteric artery occlusion followed by percutaneous low level laser irradiation of the right upper bronchus. Rats were euthanized after 4 hours of reperfusion. This study concluded that laser treatment following intestinal IRI resulted in decreased lung edema and inflammation through decreased myeloperoxidase activity and TNF-  $\alpha$  generation within the lung parenchyma.<sup>94</sup>

A limitation of our study was failure to investigate the effects of LLLT in the phases of tissue recovery and healing. Rather, our model was effective in creating repeatable and consistent small intestinal IRI that could allow for evaluation of treatment effects on the acute phases of reperfusion in local and distant tissue. Although the intestine is

possibly the most susceptible organ and the mucosal epithelium the most sensitive structure to IRI, there is capacity for rapid recovery through the processes of restitution, migration and proliferation.<sup>4,18,25</sup> Depending on the severity and duration of ischemia, the intestinal mucosa can potentially recover within 24 hours following ischemic injury.<sup>4,25</sup>

The model chosen is one that is well established for the study of bowel IRI.<sup>4,17,18,24,25</sup> The consequences of intestinal IRI are related to the duration and extent of ischemia (partial vs. complete, arterial alone vs. arterial and venous). Complete intestinal ischemia is more devastating than incomplete; however, paradoxically, partial ischemia has been reported to result in more substantial reperfusion injury compared to complete ischemia.<sup>4,23,24</sup> For example, Megison et al. compared rat models that included partial intestinal ischemia (SMA occlusion only) and complete ischemia (SMA occlusion plus collateral ligation) over various time periods and found that complete occlusion resulted in greater mortality compared to partial ischemia and that longer ischemia times (up to 90 minutes) were more devastating than shorter ischemic periods.<sup>24</sup>

Park et al. compared occlusion of the intestinal arterial and venous supply to arterial occlusion alone.<sup>23</sup> This group found that arterial and venous occlusion less than 20 minutes did not result in detectable tissue damage. Ischemia times between 20-90 minutes led to mucosal villous injury with a direct relationship between ischemia time and extent of injury; however, reperfusion did not result in perpetuation of injury regardless of the duration of ischemia. Conversely, when only arterial clamping was

performed for 40-60 minutes, reperfusion resulted in statistically significant exacerbation of mucosal injury.

The conclusions from these studies and others is that if ischemia is not severe enough to lead to detectable mucosal injury, then reperfusion is unlikely to result in notable additional damage. However, if ischemia results in severe mucosal injury, further damage will occur with reperfusion. If ischemic injury is severe enough to affect the full thickness of bowel wall, reperfusion does not seem to result in further observable injury, though bowel necrosis and perforation may be imminent.

As intestinal healing pertains to IRI, the capacity for mucosal recovery depends on the severity and duration of ischemia. The intestinal mucosa can potentially recover within 24 hours after ischemic injury.<sup>4,17</sup> Park et al. demonstrated in a rat model (SMA occlusion) that after 45 minutes of ischemia, mucosal repair was evident within 3 hours, whereas 90 minutes of ischemia required 18 hours before mucosal damage was repaired.<sup>25</sup> After 24 hours, restitution of the mucosal surface was complete. Toth et al. performed a study that chronicled intestinal recovery following 60 minutes of SMA occlusion and 1 hour, 24 hours and 30 days of ischemia in a rat model.<sup>17</sup> This group found that at 1 hour after ischemia, damage to the intestinal epithelium included lifting of the epithelial layer, villi disintegration, crypt layer destruction and transmural infarction. However, after 24 hours of reperfusion, there was no significant difference between injured rats and sham/control rats. After 30 days there were increased number of Paneth cells in previously injured rats compared to control rats but no significant difference in other histological parameters.<sup>17</sup> Thus, there appears to be a “reperfusion

injury window” when intervention following ischemia may reduce further mucosal injury. It is this window that investigators must use when studying novel treatment strategies.

Thus this model should have been ideal for studying the effects of LLLT on intestinal healing in the acute phases. We found that at the irradiance and dose used, increased local tissue injury occurred within the first few hours of ischemia, and distant organ injury was minimized. A recovery model in which the recovery process was evaluated at 24 hours would be interesting. Based on the aforementioned studies, we would expect both the laser-treated and untreated control groups in our study to have undergone recovery of the intestinal mucosa after 24 hours of reperfusion. It would be appealing to learn whether the laser treated group recovered relatively faster between 6 and 24 hours, although it is unlikely that this would be clinically relevant. The most relevant finding from our study is the potential for minimizing distant organ injury during the intestinal recovery period.

In conclusion, LLLT administered at a dose of  $0.5 \text{ J/cm}^2$  does not provide protection against acute intestinal wall damage following intestinal IRI. Additionally, LLLT appeared to induce intestinal mucosal damage in rats not subjected to IRI. However, secondary lung injury was diminished, suggesting that LLLT can modulate the systemic inflammatory response. Additional studies are warranted to examine the response to intestinal IRI and LLLT using different laser parameters, laser treatment of secondary organs such as liver and spleen, and examination of response over a longer period of recovery.

Table 3-1. Treatment groups.

Reperfusion time	TPr	TPo	C1	C2	Sham
0	N=6	N=6	N=9	N=6	N=6
1	N=6	N=6	N=8	N=6	N=6
6	N=6	N=6	N=7	N=6	N=6

TPr: Laser treatment pre-reperfusion; TPo: Laser treatment post-reperfusion (There was no significant difference between TPr and TPo; therefore, groups were pooled to form Tx group for further analysis); C1: IRI, no laser treatment; C2: Laser treatment, no IRI; Sham: no IRI, no Laser

Table 3-2. Park's score for histologic intestinal injury

Score	Histologic description
0	Normal mucosa
1	Subepithelial space at villi tip
2	Extension of subepithelial space
3	Epithelium lifts off sides of villi
4	Denuding of villi
5	Loss of villous tissue
6	Crypt infarction
7	Transmucosal infarct
8	Transmural infarct

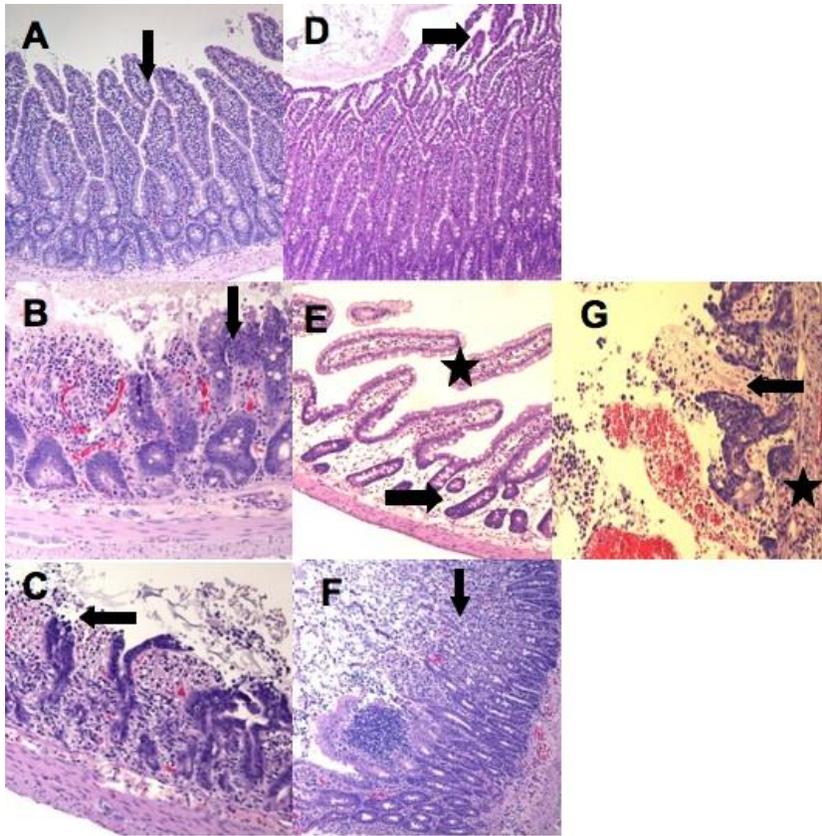


Figure 3-1. Images of intestinal injury following IRI +/- LLLT, H& E stain. A) Sham, score 0: The arrow highlights the surface of an intact villous tip covered by normal surface epithelium and represents a score of 0 or normal bowel. Magnification 100x. B) C1, 0h reperfusion, score 5: The arrow points to a blunt villous tip with regional exfoliation of the villar surfaces and loss of mucosal architecture and represents a score of 5. Magnification 400x. C) C1, 6 h reperfusion, score 8: The arrow points to an area that has lost villous structures and represents one region where the entire wall of the bowel shows early evidence of infarction and represents a variant of a score of 8. Magnification 400x. D) C2, 0h, score 2: The arrow highlights an area where the surface epithelium begins to separate from and lift off the villous tips representing extension of the subepithelial space and a score of 2. Magnification 100x E) C2, 6h, score 7: This picture represents a transmucosal infarction with the arrow highlighting edema of the lamina propria of the mucosa and the star represents an exfoliating villous tip and is a representation of a variant of a score of 7. Magnification 200x. F) Tx, 0h, score 5: The arrow points to an area of complete loss of villar architecture representing a variant of a score of 5. Magnification 100x. G) Tx, 6h, score 8: This section represents a presentation of a severe transmural infarct with the arrow highlighting a completely necrotic villous structure and the star representing an area where there is complete loss of the muscular architecture of the tunica muscularis representing the most extreme variant of a score of 8. Magnification 200x

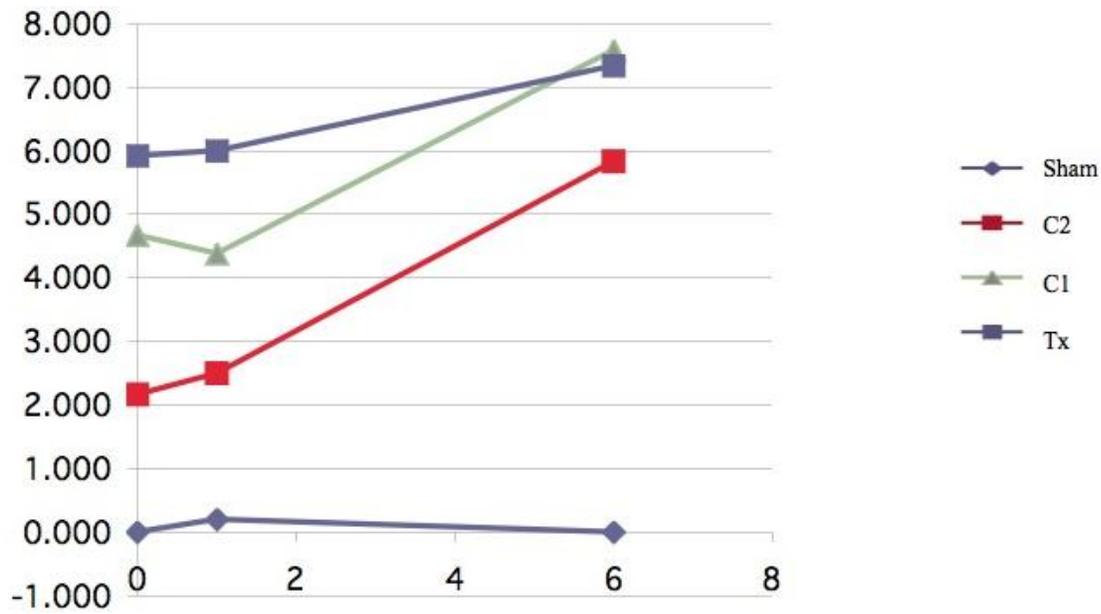


Figure 3-2. Histologic grading of intestinal injury (y axis) at 0, 1 and 6 hours of reperfusion (x axis). Groups C1, C2 and Tx were all significantly different than Sham ( $p < 0.0001$ ,  $p < 0.0001$ ,  $p < 0.001$ ). Tx animals had significantly worse injury compared to C1 at 0 and 1 hours ( $p = 0.014$ ,  $p = 0.0015$ ).

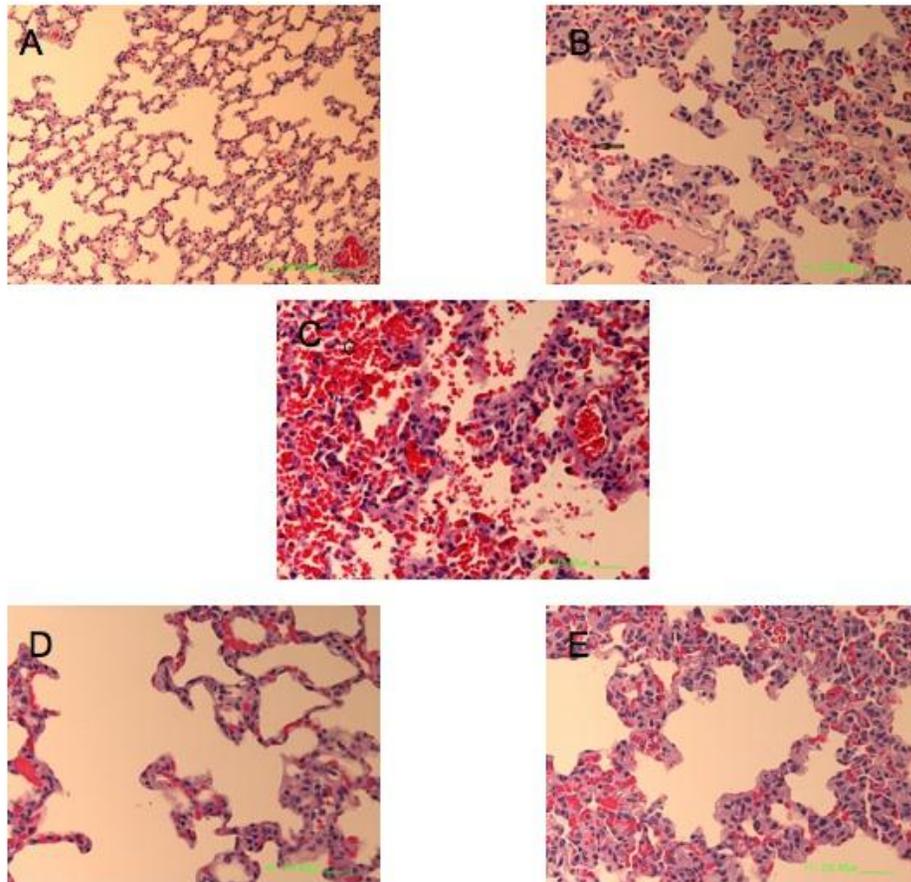


Figure 3-3. Images of lung injury following intestinal IRI +/- LLLT, H& E stain. A) Sham, score 0: Normal alveolar spaces. B) Sham, score 1: Arrow demonstrates a small area of alveolar hemorrhage. C) C1, 1 h reperfusion, score 3: Widespread hemorrhage in the alveolar spaces. D) Tx, 1 h reperfusion, score 0: Normal alveolar spaces. E) Tx, 1 h reperfusion, score 1: Small areas of alveolar hemorrhage

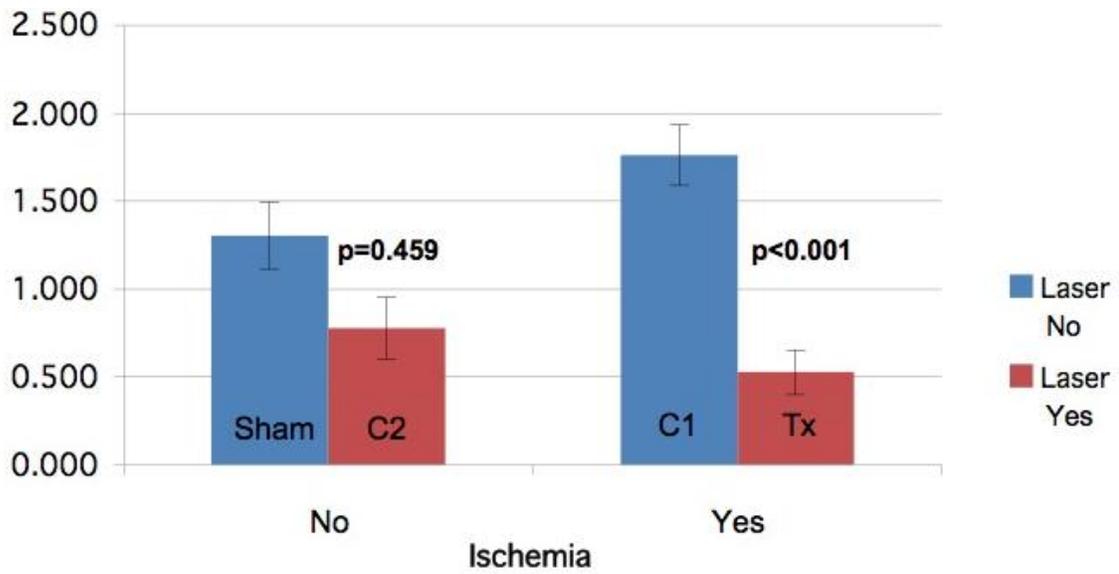


Figure 3-4. Histologic grading of lung edema (y axis) with and without IRI (x axis).  
 There is no significant difference ( $p=0.459$ ) between sham (S) and C2 groups.  
 There is a significant difference between Tx and C1 groups ( $p<0.001$ ).

CHAPTER 4  
THE EFFECTES OF LOW LEVEL LASER THERAPY (LLLT) ON CYTOKINE  
EXPRESSION FOLLOWING INTESTINAL ISCHEMIA REPERFUSION INJURY (IRI)

**Introduction**

Ischemia reperfusion injury (IRI) involves a cascade of events that include inflammation and the release of reactive oxygen species (ROS) at the site of injury.<sup>1-4</sup> Intestinal IRI commonly results in damage to secondary or distant organs such as the heart, liver, brain and lungs. The lungs are particularly sensitive to reperfusion injury.<sup>7-11</sup> Low level laser therapy (LLLT) can modulate inflammation and the production of ROS.<sup>5,6,90-94</sup> Chapter 3 described the histopathological findings in the first 6 hours following laser treatment of intestinal IRI in a rat model. LLLT (0.5 J/cm<sup>2</sup>, 35 mW/cm<sup>2</sup>, 650 nm) applied to the serosal surface of the jejunum resulted in an initial increase in intestinal mucosal injury regardless of whether or not IRI had occurred, and LLLT did not provide an apparent protective effect on the intestines. However, we did show that secondary lung injury was mitigated in animals treated with LLLT.

Previous work has shown that lung injury subsequent to intestinal IRI is mediated by a number of pro- and anti-inflammatory cytokines including tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL)-1B, IL-6, and IL-10.<sup>10,94,170,177-180</sup> For example, De Lima et al. showed in a rat model that percutaneous irradiation of the lung following intestinal IRI resulted in an increase in IL-10 expression and a decrease in TNF- $\alpha$  expression in lung tissue.<sup>94</sup> Other authors have demonstrated similar findings in the serum of rats following IRI and intervention.<sup>179</sup>

Based on the histopathologic results presented in Chapter 3, we sought to document the molecular basis for LLLT protection of the lungs in our model of intestinal IRI by measuring serum concentration of select cytokines and anti-oxidants.

### **Materials and Methods**

The details of the animal model used are described in Chapter 3. Briefly, just prior to euthanasia at the appropriate time point of reperfusion, 1 mL of blood was obtained from the caudal vena cava. Blood was immediately spun in a centrifuge for 10 minutes and the serum fraction was collected and immediately placed in a -80 °C freezer.

#### **TNF- $\alpha$ , IL-6, IL-10, Total Anti-oxidant Assay (AA)**

Serum samples were assayed in duplicate for TNF- $\alpha$  (Thermo Scientific/Pierce Biotechnology kit #ER3TNFA), IL-6 (Thermo Scientific/Pierce Biotechnology kit #ER3IL6), IL-10 (Thermo Scientific/Pierce Biotechnology kit #ERIL10), Total Antioxidant (Cayman Chemical Kit #709001). Assay protocols were followed and the final plates read on a BioTek Instruments (Winooski, VT, USA) EL-340 microplate reader or a Shimadzu (Kyoto, Japan) RF-1501 fluorescent spectrophotometer. The TNF- $\alpha$ , IL-6, and IL-10 are all competitive enzyme-linked immunosorbent assay (ELISA) procedures. The Total Antioxidant assay measures the ability of the antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] by metmyoglobin compared to that of a water soluble tocopherol analogue (Trolox), and is quantified as millimolar Trolox equivalents. The limits of detection for TNF- $\alpha$  were 31 to 2500 pg/mL and the inter and intra assay coefficients of variation were <10%. The limits of detection for IL-6 were 62.5 to 4000 pg/mL and the inter and intra assay coefficients of variation were <15% and <7%, respectively. The limits of detection for IL-10 were 16 to 500

pg/mL and the inter and intra assay coefficients of variation were <10%. The limits of detection for AA were 0.044 to 0.330 millimole (mM) and the inter and intra assay coefficients of variation were 3% and 3.4%, respectively.

Statistical calculations were performed using a computer software program (SAS PROC MIXED, SAS 9.1, Cary, NC).<sup>162</sup> The design had three factors: laser dose (0, 1), ischemia (yes or no), and sacrifice time (0, 1, 6). The response variables were: TNF- $\alpha$  (pg/mL), IL-6 (pg/mL), IL-10 (pg/mL) and AA (mmol/L). Data was analyzed using the Wilcoxon rank sum test for non-parametric data. Laser treatment pre and post reperfusion were compared by means of an unpaired t test and the Wilcoxon rank sum test. A p value <0.05 was considered significant.

## **Results**

There was no significant difference between laser treatment pre- or post reperfusion therefore all treatment groups were pooled for further evaluation ( $p>0.65$ ).

### **TNF- $\alpha$**

After 1 hour of reperfusion there was a significant increase in serum TNF- $\alpha$  levels in animals undergoing IRI and no LLLT (C1) compared to sham ( $p=0.03$ ) (Figure 4-1). After 6 hours of reperfusion this relationship still persisted but was not significant ( $p=0.11$ ). Laser treatment of intestine subjected to IRI (Tx) did not significantly decrease serum TNF- $\alpha$  levels compared to IRI without laser ( $p>0.25$ ), although there was a trend toward decreased TNF- $\alpha$  in Tx animals (Figure 4-1). Laser treatment without IRI (C2) did not increase TNF- $\alpha$  levels compared to sham at any time point ( $p>0.41$ ).

## **IL-6**

After 1 hour of reperfusion, there was no significant increase in serum IL-6 in any group (Figure 4-2). After 6 hours of reperfusion, serum IL-6 increased significantly in all animals that underwent IRI (C1 and Tx) compared to animals that did not experience IRI (Sham and C2) ( $p=0.02$ ). There was not a significant difference between laser treatment (Tx) and no LLLT (C1) in animals that experienced IRI, although there was a trend toward decreased IL-6 in Tx animals. Laser treatment alone (C2) did not affect IL-6 production compared to sham.

## **IL-10**

Serum IL-10 increased significantly ( $p<0.03$ ) following IRI at 1 and 6 hours in laser treated (Tx) and untreated (C1) animals compared to animals not subjected to IRI (Figure 4-3). Laser treatment (Tx) did not significantly influence IL-10 production compared to IRI untreated animals (C1). Laser alone (C2) did not increase IL-10 compared to sham.

## **AA**

There was no significant difference between any groups at any time point in serum total anti-oxidants (Figure 4-4).

## **Discussion**

Intestinal IRI results in the production of a number of inflammatory products that are responsible for potentiating local organ dysfunction. Furthermore, with restoration of blood flow to a previously ischemic bowel, inflammatory mediators are released the into systemic circulation and can cause deleterious effects in distant organs. The lungs are particular sensitive to reperfusion injury and inflammation characterized by vascular permeability and neutrophil accumulation is well documented.<sup>9-12</sup> The development of

acute respiratory dysfunction (ARDS) following intestinal IRI in people is associated with a 40% mortality rate.<sup>180</sup>

Tumor necrosis factor  $\alpha$  and IL-6 are pro-inflammatory cytokines that have been shown to accumulate in the intestine and mesenteric lymph node following intestinal IRI.<sup>180</sup> Interleukin 10 is an anti-inflammatory cytokine that is also produced in the acute phase of injury.<sup>94</sup> These cytokines are delivered through the venous and lymphatic systems to the lungs where they (among other cytokines and mediators such as IL-8, leukotriene B4, thromboxane B2, platelet activating factor, and ROS) modulate vascular permeability, neutrophil activation and adhesion and endothelial cell apoptosis.<sup>177</sup>

Low-level laser therapy has been widely investigated as a method of enhancing wound healing and providing pain relief.<sup>89,147</sup> Laser energy is absorbed by cytochrome c in the mitochondria of cells, leading to enhanced production of ATP. The consequences of this basic interaction are wide spread and include restoration of cell membrane pumps, increased expression of growth factors, modulation of leukocyte activity, vasodilation, decreased vascular permeability, modulation of oxidative radicals, and regulation of inflammatory cytokines and gene expression.<sup>6,91-</sup>

<sup>94,114,117,118,120,121,122,128,135,143-45</sup> Laser therapy has been shown to provide protection in skeletal, cardiac and intestinal IRI models by decreasing TNF- $\alpha$  and IL-6, up-regulating the production of IL-10, and increasing the activity of endogenous free radical scavengers.<sup>91-94</sup>

Our study found that 60 minutes of intestinal ischemia followed by 1 hour of reperfusion led to a significant increase in TNF- $\alpha$  in the serum. Reperfusion for 6 hours led to a significant increase in IL-6. These results corroborate our histologic finding of

significant lung injury following reperfusion in the same animals. However, our histologic results indicated a protective effect of LLLT on intestinal IRI induced lung injury (Figure 3-4). While there was certainly a trend toward decreased TNF- $\alpha$  and IL-6 in laser treated animals, we did not find statistically significant differences between laser treated and untreated animals (Figures 4-1, 4-2). This discrepancy may be due to a type II statistical error as there were relatively few numbers of rats in each group and wide variation in results (Figures 4-1, 4-2). Additionally, there may have been a statistical difference had we measured cytokine expression in the lung tissue rather than in the serum, as was performed by De Lima et al.<sup>94,178</sup>

We found that IL-10 increased significantly in both laser treated and untreated groups after 1 hour of ischemia and 1 and 6 hours of reperfusion (Figure 4-3), but laser did not appear to significantly influence IL-10 expression following IRI. Based on the study by De Lima et al, we would have expected laser treatment to lead to increased expression IL-10.<sup>94</sup> However, as previously stated we measured this cytokine in the serum rather than the lung tissue, where perhaps there may have been greater activity in response to IRI-induced inflammation.

An important finding from these results is that laser treatment of uninjured bowel did not lead to an increase in any of the measured cytokines. This is in contrast to our histologic findings where laser treatment resulted in statistically significant injury of the bowel compared to sham operated groups (Figure 3-2). Furthermore, laser treatment of IRI effected bowel resulted in histologically more severe damage compared to IRI without laser. Taken together, these results imply that the local tissue injury caused by laser treatment is not mediated by TNF- $\alpha$ , IL-6 or IL-10. There may be other

inflammatory mediators that are involved, but it is likely that the mechanism of tissue damage is secondary to another pathway such as free-radical induced or thermal injury.

Red and infra-red laser irradiation is known to result in the production of ROS and reactive nitrogen species as part of the photodynamic interaction with cytochrome c and the mitochondrial respiratory chain.<sup>124</sup> The generation of these free radicals in small quantities results in increased intracellular signaling and transcription of genes related to cellular division and proliferation.<sup>109,124</sup> It is possible that the histological damage seen with laser irradiation is due to either expected or excessive ROS generation following phototherapy.

Laser therapy can increase the production of endogenous anti-oxidants following IRI.<sup>93</sup> To investigate this effect we measured total anti-oxidants in the serum; however, we did not find a significant difference in any of the groups at any time point. Rather, the data had very wide ranges of values in all groups, particularly at 1 and 6 hours of reperfusion. No conclusions can be drawn from these results and we suspect that sample handling or processing may account for the widely varying data. Avni et al. utilized total anti-oxidants as an outcome measure when investigating the effects of LLLT in a skeletal muscle model of IRI and found significantly increased total antioxidant activity in laser treated groups.<sup>93</sup> However, tissue rather than serum was used in Avni's study, which may account for the difference in results between their study and ours.

The absorption of laser radiation by photoreceptors results in the generation of heat.<sup>107,124</sup> Depending on the power and energy density of the laser being used, this heat may be imperceptible and dissipated quickly, or may be substantial and lead to

thermal-induced tissue injury. While the dose of laser we delivered ( $0.5 \text{ J/cm}^2$ ) was considered low and the power output of the laser also relatively low (70 mW) the concentration of this energy in a small area (energy density) may have been high enough to result in considerable tissue heating. Measurement of heat shock proteins (HSP) such as HSP70 would be interesting as this group of molecules have been studied in other phototherapy models because of their role in repairing thermal-induced protein denaturation.<sup>181</sup> Furthermore, LLLT was shown to induce HSP70 in cardiac and skeletal muscle following IRI.<sup>93</sup> The degree of injury in laser, no IRI (C2) groups was confined to the intestinal villi and thus would be expected to recover without intervention. However, the finding of laser-induced changes in otherwise normal tissue highlights the importance of laser safety in a clinical situation.

To summarize our findings, our model resulted in significantly increased expression of TNF- $\alpha$ , IL-6 and IL-10 after intestinal ischemia and reperfusion. Animals treated with LLLT tended to have decreased production of TNF- $\alpha$  and IL-6 compared to untreated animals but these results were not significant. However, laser modulation of these inflammatory mediators may, at least in part, account for the protective effect we found on the lungs in animals that underwent intestinal IRI and were treated with LLLT. Small sample size may account for some of the failure to show statistical difference. Furthermore, repeating the cytokine and total anti-oxidant tests on lung and intestinal tissue may yield more conclusive results. Finally, the mechanism of laser-induced intestinal injury we showed in our histologic results does not appear to be mediated by TNF- $\alpha$ , IL-6 or IL-10.

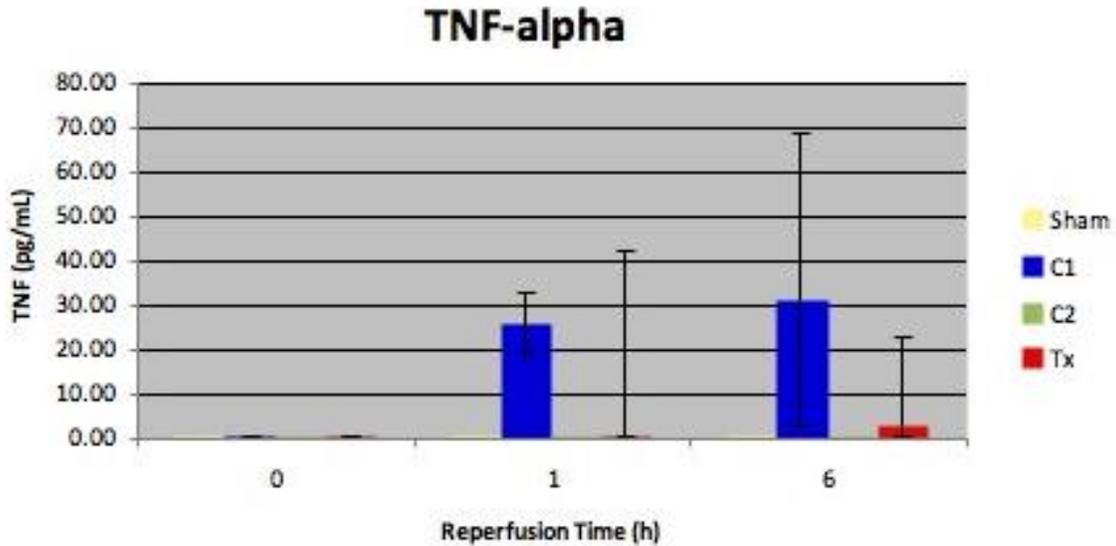


Figure 4-1. Serum TNF- $\alpha$  following intestinal IRI +/- LLLT. After 1 hour of reperfusion, TNF- $\alpha$  increased significantly in C1 compared to sham ( $p=0.03$ ). There were no other significant differences between groups.

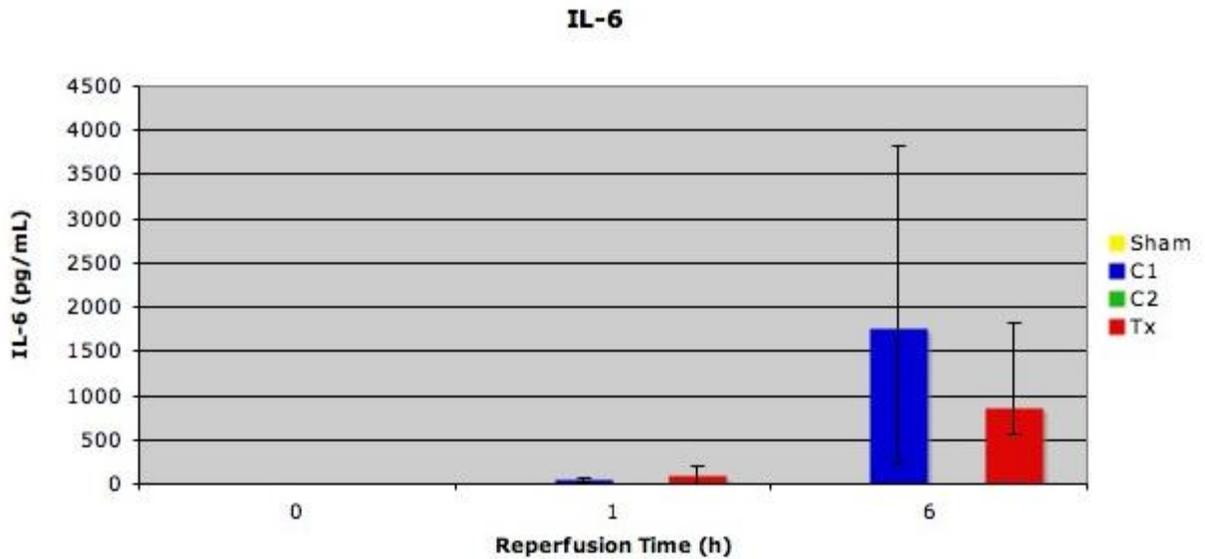


Figure 4-2. Serum IL-6 following intestinal IRI +/- LLLT. After 6 hours of reperfusion, IL-6 significantly ( $p=0.02$ ) increased in C1 and Tx groups compared to Sham and C2 groups, although there was not a significant difference between Tx and C1.

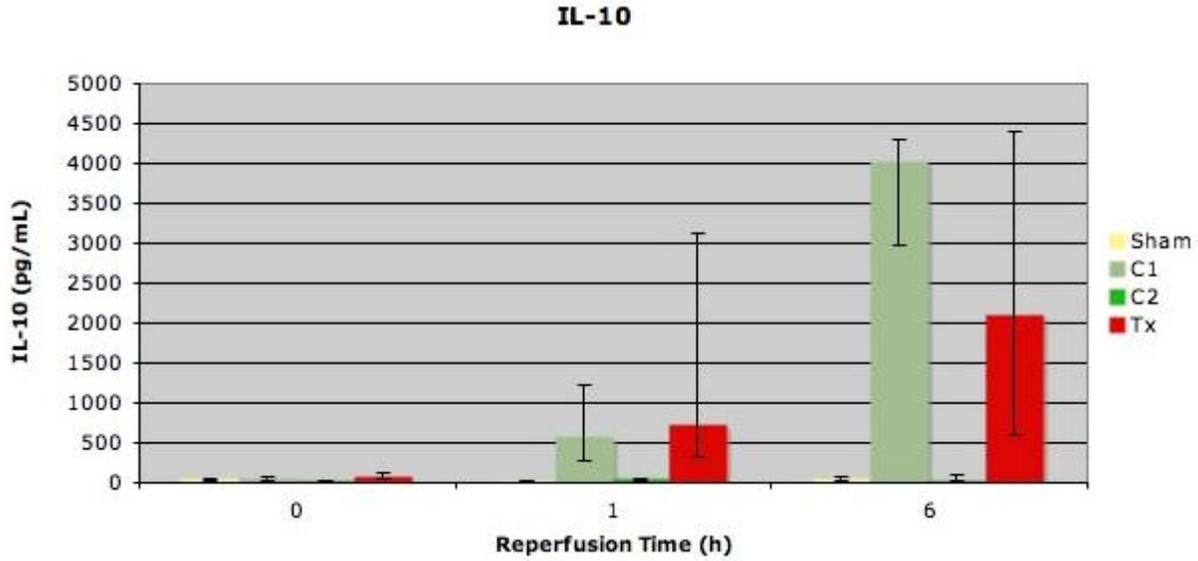


Figure 4-3. Serum IL-10 following intestinal IRI +/- LLLT. There was a significant increase ( $p < 0.05$ ) in IL-10 in C1 and Tx groups at 1 and 6 hours of reperfusion compared to Sham and C2, but no significant difference between C1 and Tx groups at either time point.

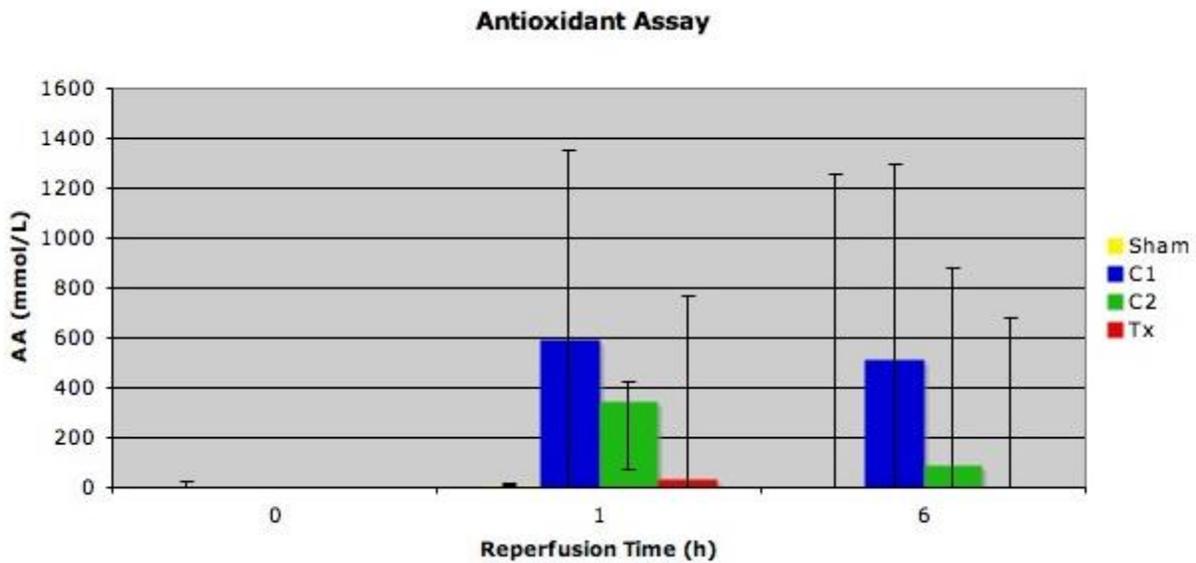


Figure 4-4. Serum total anti-oxidants (AA) following intestinal IRI +/- LLLT. There was no significant difference between plasma total anti-oxidants in any groups.

## CHAPTER 5 CONCLUSION

This study was designed to investigate the effects of a novel treatment modality in a rat model of intestinal ischemia reperfusion injury (IRI). The purpose of this research was to potentially discover a clinically applicable tool that could be used in veterinary (and human) patients to enhance intestinal healing following IRI and prevent distant organ reperfusion injury. For an intervention to be applicable, it must be one that is employed after the injury has occurred, as apposed to preventative measures such as ischemic preconditioning (IPC). The intervention must also be readily accessible, cost effective, and not cause harmful or unwanted side effects.

Low level laser therapy (LLLT) is a modality that is widely available and can be very cost effective, depending on the laser device that is purchased. Lasers are portable and could easily be used in an operating room setting with appropriate sterility measures taken. This study was the first to evaluate the effects of LLLT applied directly to the intestine, as could be performed in a clinical setting. While protective effects on lung injury were shown, the results of this study do not support the immediate integration of LLLT for management of intestinal IRI because of the potential for deleterious local tissue injury.

There are several limitations to this study, some of which have been discussed in Chapters 3 and 4, and a number of future studies that could be designed based on these results. This Chapter will review the study limitations as they relate to veterinary clinical relevance and propose future studies that could add information that was not gleaned from this work.

## Limitations of the Model

Numerous rodent models have been described for the study of intestinal IRI, with the primary differences being the degree and duration of vascular compromise.<sup>4,17,23-25</sup> The superior mesenteric artery (SMA) occlusion model used in this study is considered a partial bowel ischemia model because collateral vessels were not ligated.<sup>24</sup> This model is expected to cause substantial intestinal damage, but less severe injury than would occur with complete ischemia, allowing for assessment of reperfusion consequences.<sup>23,24</sup> The duration of SMA occlusion chosen (60 minutes) reliably results in mucosal and transmural intestinal injury severe enough to detect a difference with intervention, but with the capacity to heal within 24 hours.<sup>17</sup> Furthermore, 60 minute SMA occlusion with subsequent reperfusion leads to distant organ injury.<sup>10,11,94,177,178</sup> Thus, the model used for this study was ideal for evaluating the effects of intervention on the acute phases of intestinal healing and distant organ injury.

However, SMA occlusion may not be the most relevant model for veterinary patients. Clinically, SMA occlusion best represents primary vascular disease that is seen almost exclusively in humans. Conditions that cause intestinal IRI in veterinary patients most commonly result in both arterial and venous occlusion. However, Park et al. showed that 60 minutes of arterial and venous occlusion, representing vessel strangulation, did not lead to exacerbation of injury with reperfusion.<sup>23</sup> Had we utilized a model such as this, we would not have expected to see significant changes locally during the reperfusion period. However, this model may be well suited for more specifically evaluating the effects of LLLT or other interventions on distant organ injury.

Perhaps the most clinically relevant model is low-flow ischemic injury described by Chiu et al. in dogs and Moore et al. in horses.<sup>20,29</sup> Reduction of blood flow to 20% of

baseline can predictably lead to ischemia with subsequent reperfusion injury and represents disease conditions such as bowel distension and severe hypotension or shock.<sup>20,29</sup> This model also represents injury that occurs at the periphery of strangulated bowel, an area that is clinically relevant because it corresponds to the area where viability may be questionable based on gross inspection (as apposed to the clearly non-viable strangulated portion) and also pertains to the location of potential anastomosis if significant resection is needed. The low-flow model described in dogs and horses is not commonly used in rodents and the sequence of histologic events following ischemia and reperfusion are not well described in rats.

### **Limitations of Outcome Measures**

As discussed in Chapter 1, there are numerous methods of assessing the bowel for ischemic injury. The outcome measures used in this study were not ones that would be clinically relevant, but were designed to detect differences between treatment groups following ischemia and reperfusion.

The outcome measures used in this study included histologic evaluation of intestine, lung and liver and detection of cytokines and anti-oxidants in the serum. The primary outcome of interest was intestinal damage, which was assessed using Park's scoring system. This is a well established grading system in rats following intestinal IRI and is based on the degree of mucosal and submucosal injury.<sup>23</sup> The degree of injury seen in control (C1) animals in this study correlated well with previous studies using the SMA model.<sup>23</sup>

The second outcome of interest was the degree of secondary or distant organ damage. No significant lesions were seen in the liver; however, significant differences were seen between groups in the amount of lung edema and hemorrhage (Figures 3-3,

3-4). The grading scale used to evaluate lung injury was not a referenced system such as Park's but was developed by the veterinary pathologist who was performing the evaluation. This is a potential limitation of the study that perhaps makes the findings less robust. However, the pathologist performing evaluation and grading is highly experienced in evaluating rodent lung specimens and was blinded to the treatment groups. Ideally, additional, validated measures would have been used to corroborate the histologic findings. For example, Evans blue dye is used as a marker of microvascular permeability because it binds strongly to albumin and extra-vascular detection of the dye in tissue indicates protein extravasation.<sup>10</sup> Others have measured the amount of Evans blue dye in lung tissue following intestinal IRI and intervention.<sup>10,11,94</sup>

The injury that occurs in lungs following intestinal IRI is primarily caused by an inflammatory response characterized by neutrophil sequestration and endothelial cell adhesion.<sup>10,11,94</sup> Pulmonary neutrophil accumulation can be assessed by quantifying neutrophils seen in histologic samples and by measuring lung myeloperoxidase (MPO) activity.<sup>10,11</sup> A significant limitation of this study was the failure to assess pulmonary neutrophil accumulation.

Serum cytokine and anti-oxidant levels were measured in order to determine the mechanism of LLLT-induced local injury and distant organ protection. Failure to reach statistical significance in many of the analyses was likely due to small sample size. The addition of these outcome measures occurred after the study had begun, and therefore, animals that were sampled at the beginning of the study period did not have serum

samples available for analysis. It would have been ideal to also measure cytokine expression in liver and lung tissue in addition to serum.

### **Limitations of LLLT**

Many of the limitations associated with LLLT were discussed in Chapters 2 and 3, but there are additional factors that would be important in a clinical scenario. The amount of time it takes to deliver a chosen dose ( $J/cm^2$ ) of LLLT depends on the laser power ( $W$ ), average power output (determined by pulse frequency), and size of the treatment area ( $A, cm^2$ ). In this model, it took 10 minutes to deliver a dose of  $0.5 J/cm^2$  to the entire small intestine ( $45 cm^2$ ). Ten minutes is probably the maximum amount of time most surgeons would consider acceptable to prolong a procedure and anesthesia. The dose of laser used in this study is considered low relative to most other studies and concern would exist that it may be outside of the therapeutic window. Yet, histologic damage was seen in the intestine of animals that did not undergo IRI but were treated with laser (C2), suggesting that the dose was sufficient to cause a cellular reaction. If one was treating a much larger area (dog, horse bowel), the time it would take to deliver even a low dose of  $0.5 J/cm^2$  would be prohibitive using a laser of this power (70 mW). Therefore, in order to give the same dose in a shorter period of time, a more powerful laser would be required. The most common therapeutic lasers available commercially have an output of 500 mW- 10W, suggesting that a reasonable treatment time could be achieved. Yet, another critically important parameter of lasers is the intensity or concentration of power in a given area ( $W/cm^2$ ). This is determined by the power of the laser and the beam diameter or spot size. Delivery of laser using high power concentrated in a small area is capable of very quickly heating and damaging tissue, whereas the same power (and dose) spread over a larger area can potentially be

therapeutic. It is possible that the deleterious effects found in the intestines of group C2 rats was a result of the intensity being too high. So, if one were using a higher power laser to decrease the amount of time required for delivery, it would be essential that the intensity be lowered by using a large spot size or treatment probe. Consequently, not only do the optimal dose and intensity of laser remain to be determined in this model, but practical translation to treating larger areas using commercially available treatment probes would be required.

### **Future Studies**

Numerous studies could be designed to build upon the results of this research. A series of trials could be performed using the same model and investigating different laser parameters. For example, altering the power of the laser while maintaining the same beam diameter and treating for the appropriate time period to reach  $0.5 \text{ J/cm}^2$  would determine the effects of laser intensity on local injury. Conversely, the same laser parameters and treatment technique could be employed but the reperfusion phase extended up to 24 hours to determine whether intestinal recovery occurred more quickly in laser treated rats. Most importantly, additional investigation into the mechanism of distant organ protection is warranted. This exact study could be continued in order to increase the number of specimens available for cytokine analysis, and additional outcome measures such as Evans blue dye and MPO could be added.

Another study could evaluate percutaneous treatment of bowel following SMA occlusion and compare results to De Lima's study in which laser was percutaneously administered to the lungs in the same model. Finally, a gastric dilatation and volvulus (GDV) model could be tested to evaluate the effects of LLLT on the injured stomach and secondary organ injury, particularly the heart.

## Conclusions Reached

The conclusions of the study have been discussed in previous chapters. To summarize the novel results:

- There was no difference between laser treatment before or after the initiation of reperfusion.
- Laser treatment at the parameters studied caused histologic damage to otherwise normal intestine. This was not mediated by tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL)-6, or IL-10.
- Laser treatment of intestines following ischemia did not protect against further injury in the first 1 hour of reperfusion. In fact, intestinal injury was significantly worse in laser treated animals during this time period. After 6 hours of reperfusion, there was no difference between laser treated and untreated groups.
- Laser treatment of intestines subjected to ischemia protected the lungs from reperfusion induced hemorrhage and edema. The mechanism of this protection is likely due in part to decreased production of TNF- $\alpha$  and IL-6 in the lung.

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## BIOGRAPHICAL SKETCH

This dissertation was completed as part of Kristin's 4th degree from the University of Florida. She received her Bachelor of Science in Animal Science in 2000 and Doctor of Veterinary Medicine in 2003. She completed a one year rotating internship in small animal medicine and surgery at Veterinary Specialists of Northern Colorado in 2004. Kristin then returned to the University of Florida for a combined small animal surgery residency and master's degree program. Her Master of Science thesis was titled "The effects of intra-venous bilirubin on renal ischemia-reperfusion injury in a rat model" and she received her MS in 2006. During her residency, Kristin developed an interest in physical rehabilitation and therapeutic modalities and became a Certified Canine Rehabilitation Therapist in April 2008. Kristin completed her surgery residency in July 2008 and became a Diplomate of the American College of Veterinary Surgeons in March 2009. Following her residency, Kristin remained at UF for her PhD while also working in the Small Animal Hospital as a surgeon and developing the small animal physical rehabilitation service. She received her Ph.D. in December 2012, and now lives and works as a veterinary surgeon and rehabilitation therapist in Washington. Kristin is also a faculty member of the Canine Rehabilitation Institute and consultant for Cutting Edge Therapeutic Lasers and has lectured nationally and internationally on the topic of therapeutic lasers in veterinary medicine.