REGIONALLY GRADED INJURY FROM THE DUODENUM TO ILEUM IN HEAT AND SIMULATED ISCHEMIA

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2011

To my family; by birth, choice, or just plain luck

ACKNOWLEDGEMENTS

I would like to think Dr. Thomas Clanton for his mentorship and the opportunity to pursue a master's degree at the University of Florida. I would also like to thank all other members of the Clanton lab for their guidance, collaboration, and high spirits throughout my time spent there. Finally, I would like to thank the other members of my thesis committee, Dr. Randy Braith, and Dr. James Collins, for their suggestions and expertise.

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LIST OF ABBREVIATIONS

FD4	Fluorescein isothiocyanate (FITC)-Dextran 4000 Da; a fluorescent protein sized to mimic lipopolysaccharide (LPS) and is used to measure tissue permeability.
H + E	Hemotoxin and eosin
HS	Heat Stroke
KCl	Potassium Chloride
LPS	Lipopolysaccharide, or endotoxin; bacterial components in the intestine that stimulate immune and inflammatory responses
TFF3	Trefoil Factor 3
TLR	Toll-Like Receptors
NOC-12	3-Ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene
NLR	NOD-Like Receptors
VH/CD	Villi Height to Crypt Depth Ratio
VH/VW	Villi Height to Villi Width Ratio

Abstract of Thesis Presented to the Graduate School
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May 2011

Chair: Thomas L. Clanton
Major: Applied Physiology and Kinesiology

Heat exposure is believed to contribute to loss of intestinal barrier function due to thermal injury as well as ischemic insult. We have previously reported that regions proximal to the stomach develop greater permeability during in vitro exposure to heat. In this study we tested if the same trend was seen in an *in vitro* model model of simulated ischemia. Intestinal segments of adult mice were isolated and everted to create ~8 x 2 cm sacs. All tissues were first incubated at 37+ 0.5°C for 30 min in fresh cell culture media. Then sacs were transferred to preheated media at 42+ 0.5°C for 90 min. Permeability was measured by accumulation of fluorescent labeled dextran (4 kDa) that diffused into the sacs during heat treatment. Consistent with our previous results, multi way ANOVA showed that both treatment and region of intestine where significant determinants of permeability, with the greatest permeability seen at the duodenum, the region of the intestine closest to the stomach. As a follow up, we also examined if an *in vivo* heat stroke model would result in more prominent morphological injury to the duodenum. Anesthetized mice were exposed to control (37° C for 2 hours) (N = 6) or heat stress (HS) treatment (39.5 °C with 0.5°C increases every 30 min until core temperature reached 42.4° C) (N = 6). After 30 min of recovery, multiple regional

samples were removed, fixed, sectioned and stained with H&E for light microscopy evaluation of intestinal morphology and tissue damage. Multi way ANOVA showed that HS mice had significantly lower villi height/ crypt depth ratios (VH/CD) ($p < 0.01$). VH/CD was also highly dependent on region, with duodenum showing the greatest decrease and ileum showing the least ($p < 0.01$). HS mice also showed greater decreases in VH/CD across regions compared to control ($p < 0.01$). Histological analysis of intestines from *in vivo* experiments in HS adds additional information to our previous findings in *in vitro* experiments. In both cases, the results show that regions of the small intestine proximal to the stomach are more susceptible to damage. This has important implications regarding the origins of immunological responses to heat stroke and treatments for intestinal damage suffered in extreme conditions.

CHAPTER 1
INTRODUCTION

Between 1997 and 2006, 54,983 people were treated in United States emergency rooms for heat related illness (1). Heat stroke is the most severe category of heat illness, characterized by an elevated core body temperature above 40° C, central nervous system dysfunction, intestinal barrier dysfunction, and endotoxin translocation, resulting in an inflammatory cytokine cascade. Severe cases of heat stroke result in multi organ failure and death (2), with mortatiliy ranging from 10 to 50%.

Intestinal barrier dysfunction is a critical component to the developing pathology of heat stroke. In addition to volatile temperatures, the intestine becomes particularly vulnerable to ischemia, as splanchnic blood is shunted towards the periphery in an effort to dissipate heat (3-4). Increased sweating and dehydration result in decreased plasma volume and hypotension, further increasing ischemic injury risk. Ischemic injury manifests in a three part progression: 1) splanchnic hypoperfusion, 2) ischemia-reperfusion-mediated injury, and 3) the loss of gut-barrier function. Together, these three pathological conditions promote intestinal bacteria and endotoxin translocation into the circulatory and lymphatic systems, leading to the production of inflammatory cytokines and potential multi organ failure (5).

Our lab has been examining the effects of heat stroke-like conditions on the intestine using an *in vitro* model originally developed by Lambert et al. in the rat (6). While our lab observed the expected increases in permeability in hyperthermia, we did not see significant increases in permeability when adding tight junction openers such as: E. coli heat-stable enterotoxin, palmitoyl-DL-carnitine, and NOC-12 at 37° C. Significant increases were seen with the tight junction opener, cytochalasin D, but not to

the same levels of hyperthermia alone (7). With this finding, we extensively examined the protocol and modified it as necessary to reduce variability in environmental conditions and measurement techniques (8). After these improvements to the protocol were implemented, it was repeatedly observed that under heat treatment, intestinal permeability was significantly dependent on region (8). The more proximal an intestinal sac was to the pyloric sphincter, the greater its susceptibility to developing permeability in hyperthermia. In addition, when a best fit line was applied to permeability as a function of segment location, heated intestine had a significantly higher slope than control. (Figure 1-1) These results contrast previous work by Lambert in the rat, who found no significant difference between permeability across intestinal regions when subjected to elevated temperatures (6).

More work with this *in vitro* model was needed to determine if this phenomenon was also present in the second stressor common to heat stroke, ischemia. In addition, more testing was needed to determine if a whole animal model would display the same regional susceptibility to damage as the *in vitro* model. The purpose of this investigation was to determine if intestinal sacs were regionally susceptible to permeability in simulated ischemia and to follow this up with additional *in vivo* experiments using microscopy to examine regional damage in heat stroke.

Specific Aim 1

To develop an *in vitro* ischemia-induced intestinal permeability model in the mouse and to determine the relationship between small intestinal region and ischemia-induced permeability defects.

Hypothesis for Aim 1

H1: Simulated ischemic stress, *in vitro*, will result in a graded change in permeability that is dependent on intestinal region such that duodenal regions will be more permeable than ileal regions.

Specific Aim 2

To determine if the extent of intestinal damage in a model of whole animal heat stroke is localized to specific regions of the small intestine and to characterize the histopathology of hyperthermia-induced intestinal damage in the mouse.

Hypothesis for Aim 2

H2: The intestines from animals exposed to heat stroke will display greater damage in the duodenum compared to the jejunum and ileum.

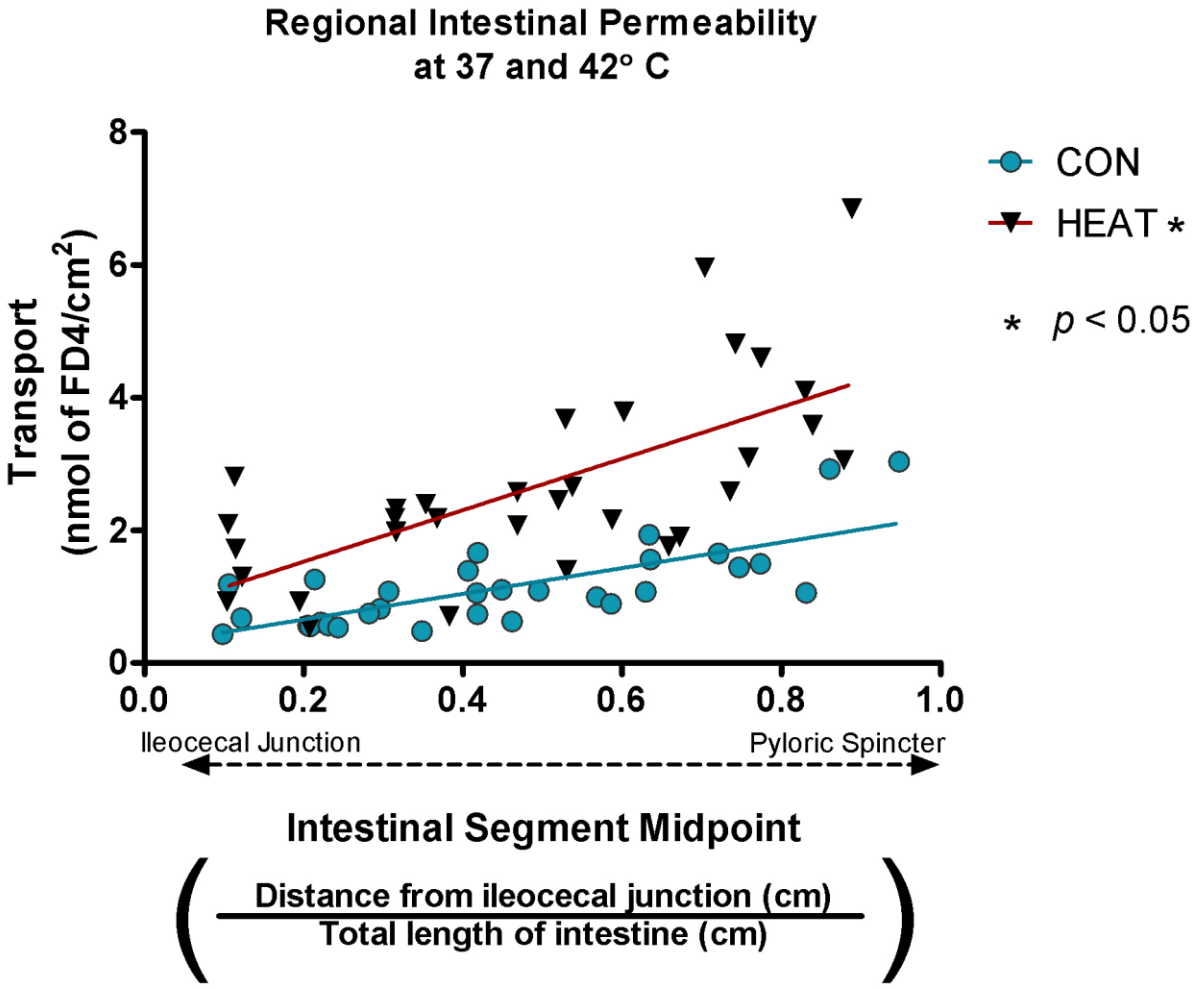

Figure 1-1. Regional intestinal permeability at 37° and 42° C. Multiway ANOVA showed that region and treatment were significant factors in determining transport ($p < 0.05$). Slopes where compared between control and heated best fit lines, and the effects of heat treatment on slope was significantly greater than control ($p < 0.03$).

CHAPTER 2
LITERATURE REVIEW

Healthy Intestinal Barrier Function

The intestine is believed to play an integral role in the development of heat stroke, and is particularly susceptible to acute heat stroke and ischemia (9-10). In addition to absorbing nutrients, the healthy intestine acts as a barrier between sterile blood and non-sterile luminal contents such as pathogenic bacteria or digestive enzymes. The intestinal barrier is equipped for this task with multiple defenses, including a mucosal layer, an epithelial cell layer held together by tight junctions, and a variety of vascular, lymphatic and immune cells such as tissue macrophages, dendritic cells and resident lymphocytes (11).

Mucosal Layer

The first layer of the mechanical barrier is a thick mucosal layer which slows down bacterial penetration, increases villi lubrication, and generally separates the epithelial cells from digestive contents inside the lumen. A thick mucosal layer will also prevent over stimulation of immune cells (12). Mucins, proteins secreted by goblet cells, compose the majority of the mucosal layer. In humans, goblet cells produce approximately three liters of mucin per day, ensuring a constant renewal of mucosal protection (13).

Epithelial Layer

Below the mucous layer lies the epithelium, a single squamous cell layer placed directly between the sterile, internal body and the bacterial-laden intestinal contents (11). The epithelium is responsible for nutrient transport and bacterial sampling while still keeping intestinal contents secluded to the lumen. To accomplish this task, this

sheet of epithelial cells is held tightly together by tight junctions (11). The effect of tight junctions is great enough to even maintain the polarization of the epithelial cells and their membranes into basal and luminal zones, each with unique transport proteins and phospholipid ratios. However, tight junctions remain dynamic and are equipped with myosin light chains that act as the regulatory site for the cytoskeletal junctions that function as gate keepers. When myosin light chains are phosphorylated, they contract and the tight junctions are pulled open, and small substances pass paracellularly (14-15).

Upon the surface of the epithelium, there are a variety of germ-line encoded pattern recognition receptors that capture bacteria, translocate them internally, and promote the production of pro-inflammatory cytokines (16). Epithelial cells also have the ability to take up antigens through fluid phase endocytosis and receptor-mediated endocytosis using various receptors (12). Initially, it may seem counterintuitive that the epithelium is equipped with multiple ways to promote a “leaky gut”. However, these pathways serve a vital function to the immune system. Pattern recognition receptors include the families of toll-like receptors (TLR) and NOD-like receptors (NLR) (16). Mice deficient in TLR and NLR exhibit delayed or diminished wound healing when confronted with mucosal injury, including acute DSS colitis (16). For example, mice deficient in TLR-2, will lack the ability to produce goblet cell-derived TFF3, a mediator of epithelial migration and anti-apoptosis (17). However, the phenotype of mice with deficiency in TFF3 can be reversed with the addition of a synthetic TLR2 agonist (16). Additionally, simulation of the NLR cryopyrin has been shown to promote the production of IL-18, a

cytokine which has been shown to preserve epithelial barrier integrity during the early phase of wound healing in acute DSS colitis (16).

Immune Cells

If bacteria pass the epithelium, they will encounter gut-associated lymphoid tissue. Gut-associated lymphoid tissue is considered to be the largest immunological organ of the body, filled with B cells, T cells, granulocytes, mast cells, macrophages and dendritic cells, ready to signal an inflammatory cascade and destroy foreign bacteria (11-12). When LPS interacts with the surface TLRs on these immune cells, phagocytosis and the capacity to present antigens to T cells is enhanced (17).

Overview of Heat Stroke

Heat stroke is a life threatening condition, characterized by an elevated core body temperature above 40° C accompanied by central nervous system dysfunction, intestinal barrier dysfunction, endotoxin translocation, and a resulting inflammatory cytokine cascade (2). However, survival rates are not dependent on temperature alone, as decreased survival is also dependent on the occurrence of preceding inflammatory episodes (18). Heat stroke has multiple mechanisms of injury that contribute to multiple organ dysfunction, including thermal damage to cellular components, increased free radical production, acidosis, ischemia, and intestinal barrier dysfunction.

Thermal Damage

There is no specific core temperature threshold that marks a guaranteed onset of heat stroke; the initial onset of heat stroke has been reported to fall within a range of 40 - 43° C, specifically in the 41 - 42° C range (19). Heat exposure of 40.5° C for 60 minutes results in scattered neuronal death of 6% (20). A 20 second exposure to 42° C is the projected thermal threshold required for the initial onset of lipid bilayer

denaturation and necrosis in cell culture (21). The lipid bilayer is sensitive to stress and increases in cell membrane fluidity at 42° C have been shown to promote the heat shock response well before protein denaturation (22). At 60 minutes of 42° C exposure, the blood-brain barrier begins to break down (20). Exposure to 43° C for 10 - 60 min will result in single and double strand breaks of newly synthesized DNA (20). Prolonged heat exposure may also result in dysfunctional enzyme kinetics, leading to impaired metabolism or DNA synthesis (20). Protein degradation is exhibited at 40° C in rodent cells and 41 - 42° C in human cells, further limiting the body's ability to function in the hyperthermic environment. When 5 - 10 % of cellular proteins are denatured, the cell will undergo apoptosis (23).

Splanchnic Ischemia

In heat stroke, ischemia is initially induced when blood is shunted from the intestine and other visceral organs to the periphery in order to dissipate heat (3-4). This compromises tissues such as the liver and intestine, which both show increased hypoxia markers when exposed to heat stress (24). During hyperthermia and exercise, splanchnic blood flow can be reduced by as much as 70% (4). Dehydration and reduced blood volume due to increased sweat loads can lead to hypotension, mucosal acidosis, hypovolemia, as well as decreased renal function (5, 19). These tissues are eventually reperfused during recovery which contributes additional free radicals to the heat stressed environment. Ischemia-reperfusion injury is well known for causing increased intestinal permeability, activating an inflammatory response through TLRs, mitochondrial damage, epithelial necrosis, and potential multi-organ failure (17).

Free Radical Production

Multiple tissues increase free radical production as a result of heat stress including: brain (25), intestine (26), muscle (27) and splanchnic blood (28-29). Together, hyperthermia and ischemia promote an environment with altered pH and increased free radical production. It is theorized that lactic acidosis seen in heat stroke is brought on by the onset of local hypoxia due to poor perfusion and hypovolemia (19). The body then overcompensates for this metabolic acidosis, and respiratory alkalosis follows (30). Mucosal acidosis of the intestine leads to increased free radical production. Increased free radicals contribute to intestinal permeability and antioxidant therapy has been shown to reduce symptoms of intestinal barrier dysfunction. A xanthine oxidase inhibitor has been shown to reduce heat stress induced intestinal permeability (27) and supplementation of the antioxidant vitamin C decreases the elevated plasma endotoxin associated with exertional hyperthermia (31).

Intestinal Barrier Dysfunction

There are numerous reports that increased thermal load (6, 32-34) and mucosal ischemia-reperfusion (17, 35) each separately lead to increased intestinal permeability as well as endotoxin translocation into the blood stream. As previously stated, heat stroke combines these stressors. When excessive endotoxin due to increased intestinal permeability cannot be cleared by the gut-associated lymphoid tissue, liver, or kidneys, the intestinal endothelium is activated and neutrophils are recruited (36). This is the start of a four step downward spiral towards multi-organ failure, where a leaky gut leads to inflammation, and in return, inflammatory cytokines further promote a leaky gut through tight junction opening and endothelial apoptosis.

Step 1: Initial Onset of Injury

Heat stroke has multiple mechanisms of intestinal injury: thermal damage to cellular components, enzymatic dysfunction, acidosis, increased reactive oxygen species, and ischemia. Cells are compromised; reduced blood flow leads to reduced waste removal and reduced oxygen delivery.

Step 2: Intestinal Permeability and Endotoxin Translocation

Poor environmental conditions and/or activation of pro-inflammatory pathways induce the opening of tight junctions. When tight junctions open, the gut becomes permeable to endotoxin, the lipopolysaccharide (LPS) components of gram negative bacteria. LPS is translocated across the epithelium and into the underlying lymphatics of the intestine as well as the blood stream (11).

Step 3: LPS Induced Inflammation

Ischemia reduces renal blood flow and glomerular filtration rate, which reduces the rate that LPS can be cleared. LPS circulates and docks with the toll-like receptors on inflammatory cells and other cells such as muscle cells. LPS activates TLR-4 receptors on a variety of cell membranes and this leads to stimulation of the NF-κB pathway (12) and other stress-signaling pathways. In response, inflammatory cells produce pro-inflammatory cytokines. In particular, heat stroke creates significant increases in circulating IL-1α, IL-1β, IL-6, IL-10, and TNF-α (19).

Step 4: Continued Cytokine Production, and Intestinal Damage

The same pro-inflammatory cytokines that are produced by inflammatory cells in response to endotoxin exposure will also promote additional endotoxin translocation. For example, TNF-α (14, 37), IFN-γ (37-38), IL-1β (39) have been shown to promote the opening of tight junctions through myosin light chain phosphorylation. In particular,

TNF-α is responsible for phosphorylating myosin light chain kinase via nF-KB signaling (37, 40). The presence of TNF-α and IFN-γ upregulates production of myosin light chain kinase protein and gene activity as well (37, 40). Phosporylation of the myosin light chain kinase opens the tight junction via myosin-actin interactions on the tight junction cytoskeleton proteins. With lengthy exposures to IFN-γ + TNF-α or IFN-γ, epithelial cells also undergo apoptosis, promoting more intestinal barrier dysfunction (41). Pro inflammatory cytokines promote a leakier gut, which promotes endotoxin translocation, which promotes pro inflammatory cytokine production from immune cells.

Previous Work Examining Regional Permeability

Previous work examining regional permeability at 37° C is conflicting. Nejdfors et al. examined permeability in human, pig, and rat intestines using Ussing chambers. In man and pig, no significant differences in intestinal permeability to Fluorescein isothiocyanate (FITC)-Dextran 4000 Da (FD4) between jejunum, ileum, proximal, or distal colon were seen. In rat, distal colon permeability was significantly lower than the jejunum (42). Nejdors et al. conflicts with Jezyk et al. who showed higher permeability in the colon than jejunum in rabbit, dog, and monkey at 37° C (43). Pantzar et al. also observed greater permeability in the distal small intestine (20 to 5 cm proximal to the caecum) compared to the proximal small intestine (5 to 20 cm distal to the pylorus) (44). These studies primarily examined the regional differences between the small intestine and the large intestine, and no duodenal measurements were taken.

It has been repeatedly shown that heat stress increases permeability in cellular (32-33), in vitro (6), and whole animal in vivo models (34). The scientific community has repeatedly shown that heat stroke, both exertional (45-47) and non-exertional (34), will

result in increased plasma LPS concentrations. Although measuring plasma LPS can signify endotoxin translocation, plasma LPS measurements alone cannot specify where the LPS came from. There is a distinct lack of studies examining regional intestinal permeability in hyperthermia or heat stroke. Lambert et al. reported that there were no significant differences between duodenum, jejunum, ileum, or colon in the rat under hyperthermic or normal conditions (6). Jejunal histology sections however exhibited jejunal endothelial damage, but no duodenal or ileal histology measurements were reported. Our lab was not able to reproduce these results in the mouse and found that proximal regions were significantly more susceptible to hyperthermia than distal regions in *in vitro* hyperthermia conditions.

CHAPTER 3
METHODS AND MATERIALS

Chemicals Used

Medium 199 (Cellgro), L-Glutamine (Lonza), sodium bicarbonate (Acros Organics), Potassium Cloride (Fisher Scientific), DL-Lactic Acid Lithium Salt (Sigma), fluorescein isothiocyanate (FITC)-dextran 4 kDa (FD4, Sigma Aldrich), pentobarbitral (Sigma)

In Vitro Simulated Ischemia Model

Preparation of Treatment Chambers

Ischemic treatments were modeled to mimic porcine conditions of reduced intestinal blood flow, where clear relationships between reductions in blood flow and tissue O_2 CO_2, pH and K^+ have been established (48). We defined moderate ischemia as equivalent to a 50% reduction in blood flow, which was found to result in 20 mm Hg PO_2, 80 mm Hg PCO_2, 5 mM KCl, and 2.5 mM lactic acid in pigs. Severe ischemia treatment was defined as a an 80% reduction in blood flow and defined to be 35 mm Hg PO_2, 60 mm Hg PCO_2, 8 mM KCl, and 6 mM lactic acid (48). In treatment chambers, medium 199, a common cell culture media that includes amino acid substrates, was adjusted by the addition of KCl and lactic acid. Appropriate oxygen, carbon dioxide, and nitrogen mixtures were obtained in tanks and concentrations were tested via an oxygen electrode system (Oxygraph) and a fyrite CO_2 detector system, a chemical system used to calibrate tissue culture hood CO_2 levels. From this, we determined that the tank gas concentrations needed to reach our target PO_2 and PCO_2 levels were 2.9 % O_2, 11.2 % CO_2 for severe ischemia and 4.9 % O_2 and 8.5 % CO_2 for moderate ischemia. All tanks were within +/- 0.7% of the targeted gas concentrations. pH was analyzed with an

AB15 pH meter (Fisher Scientific), accurate to two decimals. All treatment chambers are bubbled for at least 30 minutes before experiments to ensure pH equilibration and gas saturation.

Animal Treatment and Gut Sac Preparation

Adult C57Bl6 mice (25-35 g) were treated according to protocols approved by the University of Florida Institutional Animal Care and Use Committee. Animals were given food and water *ad libitum* and housed at Shands Hospital. After euthanasia by carbon dioxide asphyxiation, the entire intestine was immediately removed and placed in Medium 199 prepared with L-Glutamine and bubbled with 95% O_2 and 5% CO_2. Gross mesentery, cecum, and large intestine were removed. Remaining mesentery was carefully trimmed from the small intestine. Once freed from the mesentery, luminal contents were removed by perfusion of 5 ml of oxygenated medium 199. The small intestine was then inverted over a 20 µl glass capillary tube. 2-3 cm intestinal sacs were filled with oxygenated media 199, tied with 2-0 sutures, and labeled. Care was taken to ensure the same hydrostatic pressure was applied to each sac during preparation (≈2-3 cm H_2O). Length measurements were taken at each suture site to determine the position of the tissue sample. When all sacs were prepared, they were collectively transferred to continually oxygenated chambers of 7.5 ml medium 199 in a 37º C water bath for 30 minutes.

After the intestinal sacs were acclimated to 37º C, the sacs were randomly assigned to one of four treatment chambers, control, moderate ischemia, severe ischemia, and hypoxia (Table 3-1); each contained 7.5 ml of medium 199 and a high molecular weight fluorescent marker, 4 kDa fluorescein isothiocyanate-dextran (0.3 mM;

FD4). While well below the average molecular weight of endotoxin (15-20 kDa), this molecular weight is in range the lipid component of endotoxin (2-4 kDa) (49). Intestinal sacs then were subjected to 37º C treatment for 90 minutes.

Data Analysis and Statistics for In Vitro Experiments

The sacs were removed, their contents emptied into preweighed tubes, and the surface area of the intestinal sac measured. Permeability was defined as transport of nmoles of FD4 per cm^2 using the following formula:

$$(Concentration_{serosal\ fluid} \times Volume_{serosal\ fluid}) \div Mucosal\ surface\ area$$

This method was described by Lambert et al. for the rat (6) but was adapted for mouse and modified to improve reproducibility. Volume was determined by the weight of intestinal contents collected, assuming the density of buffer = 1g/ml. Mucosal surface area was determined by compressing the sample under a plastic sheet with 30 g of weight, and measuring the area in cm^2. Concentration was determined by measuring the fluorescence of the sample and comparing it to a standard curve. Changes in permeability were determined using Analysis of Variance (ANOVA) or Student's t test for unequal variance (GraphPad Prism). Differences in variance between groups were evaluated using Fisher's F test (SASJMP). Multiple regression analysis (ANCOVA) was used to stratify potential sources of variance due to both categorical and continous variables (SASJMP). The slopes of linear regressions in control and ischemic treatments were statistically compared using t-tests for differences in slope and intercept (GraphPad Prism). All results are reported as means ± SEM; $p < 0.05$ was considered to be statistically significant.

In Vivo Heat Stroke Model

Experimental Procedure

Animals were weighed and gavaged with 10 µl/g of body weight of 20.8 mmol FD4. Then the animal was injected with 10 µl/g of 10% pentobarbitrol. Supplemental anesthesia was given throughout the experiment as 0.1 cc of 10% pentobarbitol per injection, as needed. When the mouse was fully anesthetized, a thermistor was inserted anally and two electrodes were placed subcutaneously to monitor heart rate. The mouse was placed into a heated chamber where it underwent a control or a heat treatment. The core temperature of control mice was aservo-controlled to keep core temperature at 36.5 ºC (resting day time temperature of mice, which are active only at night time). Mimicking temperatures of previous work by Leon et al. (50), in unanesthetized mice, heated mice were subjected to an initial temperature of 39.5 ºC for 30 minutes and for each subsequent 30 minutes, the temperature was raised 0.5 ºC until the internal temperature of the mouse reached 42.4 ºC, at which point the mouse was taken out of the environmental chamber and allowed to recover or a 30 minute recovery period. Animals that expired within the procedure due to respiratory failure were (≈30%) excluded from the final analysis because of the need for matching times in conditions between groups.

At the end of recovery, one last supplement of 0.1-0.33 cc 10% pentobarbitrol was administered before harvesting blood, intestine, soleus, and hind limb samples. Heparnized or Ca^{+2} chelated blood was centrifuged at 5000 rpm for 10 minutes and the supernatant was collected to examine the concentration FD4 marker in the blood (an indication of barrier dysfunction). Intestines were stored in 4% formalin at 4° C and transverse sections were cut from the duodenum, jejunum, and ileum for later

hematoxylin and eosin staining. To ensure consistency in samples chosen, mesentery was removed from the small intestine to a point where the intestine could be straightened without strain. Then the duodenum was cut 1 cm below the pyloric sphincter; the jejunum was cut half way between the pyloric sphincter and the ileocecal junction, and the ileum sample was cut 2 cm above the ileocecal junction.

Data analysis and Statistics for Histological Analysis from Intact Animals

Histology slides of the small intestine were graded according to the method by Chiu et al, modified to examine individual villi (Table 3-2) (51). Two trained raters separately graded each slide (the author and Neil Phillips). All grading was blind; neither rater knew which samples corresponded to which treatments or intestinal section. All slides that raters differed in more than one average grade were re-blinded and graded a second time over one month after the initial ratings. Each slide had multiple cuts of the same sample, thus raters chose one representative cut based on the following factors: the least number of staining artifacts, the least number of cutting artifacts, and the most complete section of intestine. For the chosen cut, one villus without artifacts was chosen at random as a starting point. This villus and every fourth villus thereafter was graded and measured until a total of 10 measurements were made, or in the case of a large number of cutting artifacts, until no more villi without artifacts were available to measure. Villus blunting and swelling were graded by the use of a crypt depth/villus height ratio and a villus width/villus height ratio, two measurements commonly used to determine brush border integrity (52-55).

Table 3-1. Simulated Ischemia Conditions

Treatment	% O_2	% CO_2	% N_2	Lactic Acid (mM)	KCl (mM)	Average pH
Control	95	5	0	0.0	5	7.30 ± 0.029
Moderate Ischemia	5	8	87	2.5	5	7.07 ± 0.006
Severe Ischemia	3	11	86	8.0	8	6.94 ± 0.009
Hypoxia	0	5	95	0.0	5	7.41 ± 0.042

Gases were premixed in tanks that would raise the buffer to corresponding gas levels when bubbled through the media. All partial pressure oxygen levels listed were tested in gas-equilibrated buffer with an Clark oxygen electrode at 37 °C and carbon dioxide gas levels where checked with fyrite at room temperature. Average pH values were experimental values measured in individual baths.

Table 3-2. Intestinal Ischemic Damage Grading System

Grade	Villus Appearance
0	Normal mucosal villus.
1	Development of subepithelial space, usually at the apex of the villus.
2	Extension of the sub-epithelial space with moderate lifting of epithelial layer from the lamina propria.
3	Massive epithelial lifting down the sides of the villus. Tip of villus is ulcerated.
4	Denuded villus with lamina propria and dilated capillaries exposed and digested.
5	Complete digestion and disintegration of lamina propria; hemorrhage and ulceration.

Chiu's intestinal ischemia damage grading system (51) was modified to grade one villus at a time out of a random sample of villi from the slide. Previously, this grading system was taken as a subjective measurement of the "entire slide" at once. No modifications were made to the categories of damage specifications.

CHAPTER 4
RESULTS

In Vitro Intestinal Ischemia Model

Intestinal segments treated under hypoxic, severe ischemic, or moderate ischemic conditions had significantly higher permeability than control segments when the responses of all of the segments from each animal were averaged (Figure 4-1). Moderate ischemic, severe ischemic, and hypoxic conditions had no significant differences between each other. Multi-way ANOVA showed that region, treatment, and their crossed effects were significant. (Region and treatment each had p value < 0.0001, crossed effect $p = 0.02$). A post hoc Tukey's HSD test showed that oxygenated control segments had statistically lower permeability than all other treatments ($p < 0.05$). As shown in Figure 4-2 (inset), when the permeability data is expressed as a function of location along the intestine, best fit lines across treatments showed a significant difference in slopes between oxygenated treatment and moderate or severe ischemia treatment ($p < 0.05$), and a significant difference in intercepts between oxygenated treatment and hypoxic treatment ($p < 0.0001$).

In Vivo Heat Stroke Model

Villi Morphology

Averages of control and heated villi heights, crypt depths, and villi widths can be seen in Table 4-1 and histology examples of region and treatment can be seen in Figure 4-3. Heated mice had significantly lower villi height/crypt depth ratios ($p < 0.0001$), lower villi height/villi width ratios ($p = 0.0047$), and higher average damage grades across all regions ($p = 0.0007$) (Figure 4-4). From duodenal to ileal regions, each region had a distinct graded response; duodenal regions exhibited higher average damage

grades than regions distal to the stomach ($p < 0.0001$) (Table 4-2 and Figure 4-4). Villi height/crypt depth ratios (Figure. 4-5) were highly dependent on region ($p < 0.0001$), with the duodenum having the highest and the ileum having the lowest. However, a crossed effect between villi height/crypt depth ratios and heat treatment was also statistically significant ($p = 0.0007$), showing that heat treatment resulted in a greater reduction in villi height/crypth depth ratios in the duodenum than in all other regions. In addition, there was a significant crossed effect between region and treatment; duodenal regions were more susceptible to heat damage than ileal or jejunal regions ($p = 0.0328$). Villi height/villi width ratios also were highly dependent on region ($p = 0.0133$), but no crossed effect between region and heat was exhibited.

Intestinal Permeability

Student t tests showed that heated mice had significantly higher blood FD4 concentration ($p < 0.05$); however an outlying point in the control group had to be removed prior because this point was greater than 15 standard deviations from the mean. Heat and control treatment groups were determined to have unequal variances of blood FD4 concentration with an unequal variances test. Therefore, a nonparametric Wilcoxon/Kruskal-Wallis test was used and, heated mice had significantly higher concentrations of FD4 in their blood samples ($p = 0.0344$).

Table 4-1. Average Villi Measurements across Treatment and Region

Treatment	Villi Height (µM)	Crypt Depth (µM)	Villi Width (µM)	VH/CD	VH/VW
Duodenum Control	555 ± 21	103 ± 4	138 ± 14	5.46 ± 0.32	4.39 ± 0.72
Jejunum Control	330 ± 26	118 ± 5	83.5 ± 6.0	2.80 ± 0.16 ‡	4.15 ± 0.62
Ileum Control	194 ± 17	93.9 ± 6.0	94.4 ± 2.8	2.05 ± 0.10 ‡	2.04 ± 0.13
Duodenum Heat	318 ± 31	102 ± 10	161 ± 10	3.23 ± 0.36 ‡	1.97 ± 0.12*
Jejunum Heat	249 ± 35	101 ± 8	91.0 ± 10.2	2.42 ± 0.19 ‡	3.05 ± 0.76
Ileum Heat	168 ± 19	93.9 ± 6.6	109 ± 8	1.78 ± 0.18 ‡	1.60 ± 0.25**

* Graphical representation of values can be seen in Figure 4-5 and Figure 4-6. Control duodenum exhibited higher VH/CD (villi height/crypt depth) ratios over all other combinations of region and treatment ($p < 0.0001$) and higher VH/VW (villi height/villi width) ratios in comparison to heated duodenum. * ($p < 0.05$) when compared to control duodenum. ** ($p < 0.01$) when compared to control duodenum. ‡ ($p < 0.0001$) when compared to control duodenum

Table 4-2. Frequency of Specific Grades of Injury across Region and Treatment

Treatment	Percentage of villi at specific grades					
	0	1	2	3	4	Average Grade
Duodenum Control	32.9 % ± 10.5	30.8% ± 8.6	18.1% ± 4.5	15.6% ± 9.2	2.5% ± 2.5	1.24 ± 0.37 †
Jejunum Control	42.7% ± 9.3	41.3% ± 6.5	16.0% ± 9.5	0% ± 0	0% ± 0	0.73 ± 0.18 ‡
Ileum Control	58.3% ± 9.8	35.4% ± 8.1	4.4% ± 2.4	1.9% ± 1.2	0% ± 0	0.50 ± 0.12 ‡
Duodenum Heat	1.9% ± 1.2	1.7% ± 1.1	39.0% ± 10.6	50.8% ± 9.3	6.7% ± 3.3	2.59 ± 0.10
Jejunum Heat	27.7% ± 10.5	34.8% ± 5.6	23.3% ± 6.9	14.2% ± 6.0	0% ± 0	1.24 ± 0.28 †
Ileum Heat	60.0% ± 17.3	25.8% ± 9.8	13.3% ± 7.7	0.8% ± 0.8	0% ± 0	0.55 ± 0.26 †

* Graphical representation of these values can be seen in Figure 4-4.Heated duodenum exhibited higher damage scores over all other combinations of region and treatment. † ($p < 0.001$) when compared to heated duodenum. ‡ ($p < 0.0001$) when compared to heated duodenum.

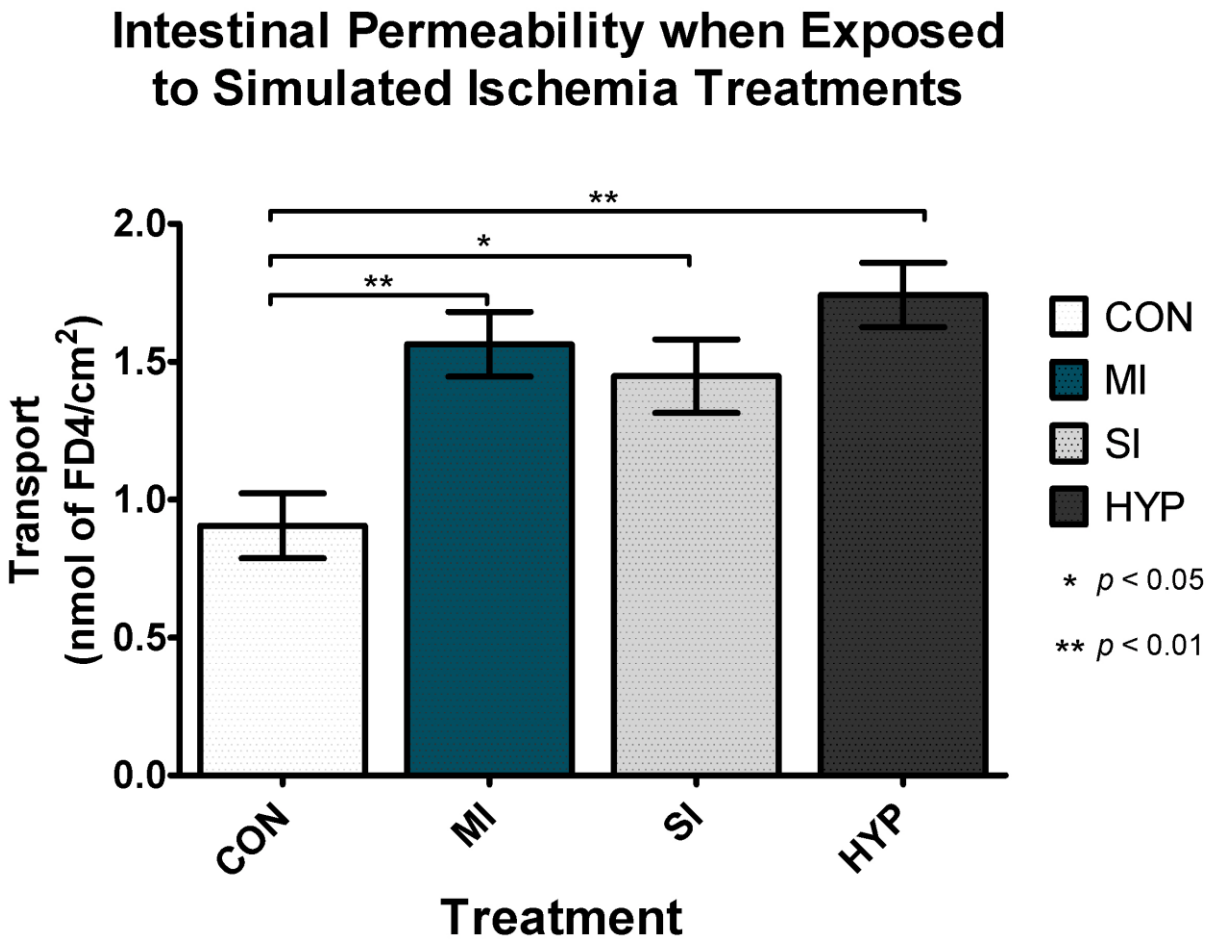

Figure 4-1. Intestinal permeability when exposed to simulated ischemia treatments. Control treatments had significantly lower FD4 transport than all other treatments. There was no statistical difference between moderate, severe, or hypoxic treatments. *($p < 0.05$) significant increase in intestinal transport of FD4 compared to control treatment. **($p < 0.01$) significant increase in intestinal transport of FD4 compared to control treatment.

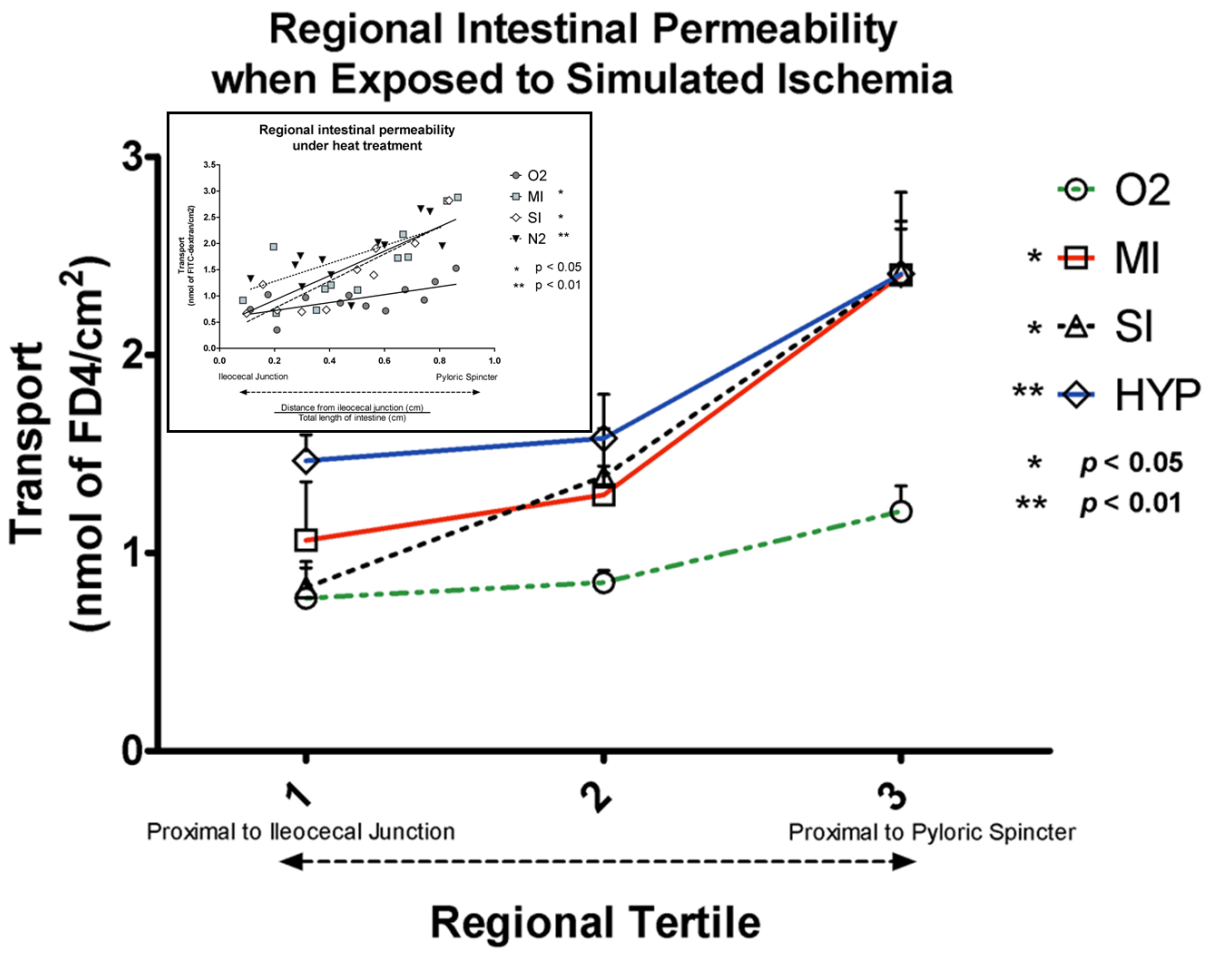

Figure 4-2. Regional intestinal permeability in different gradients of ischemia. Region was defined by measuring the midpoint of the intestinal segment from the ileocecal junction and dividing by the total length of intestine. In this graph, data were grouped within the three segment areas. In the inserted small graph, raw data were expressed from individual locations of the intestine. All treatments exhibited a trend of increased intestinal permeability in intestinal segments proximal to the Pyloric Spincter. *Moderate and severe ischemic treatments exhibited in the significantly higher linear slopes (inserted graph) compared to control treatment ($p < 0.05$). **Hypoxic treatment exhibited a significantly higher intercept compared to control treatment ($p < 0.01$).

Representative histology by region and treatment

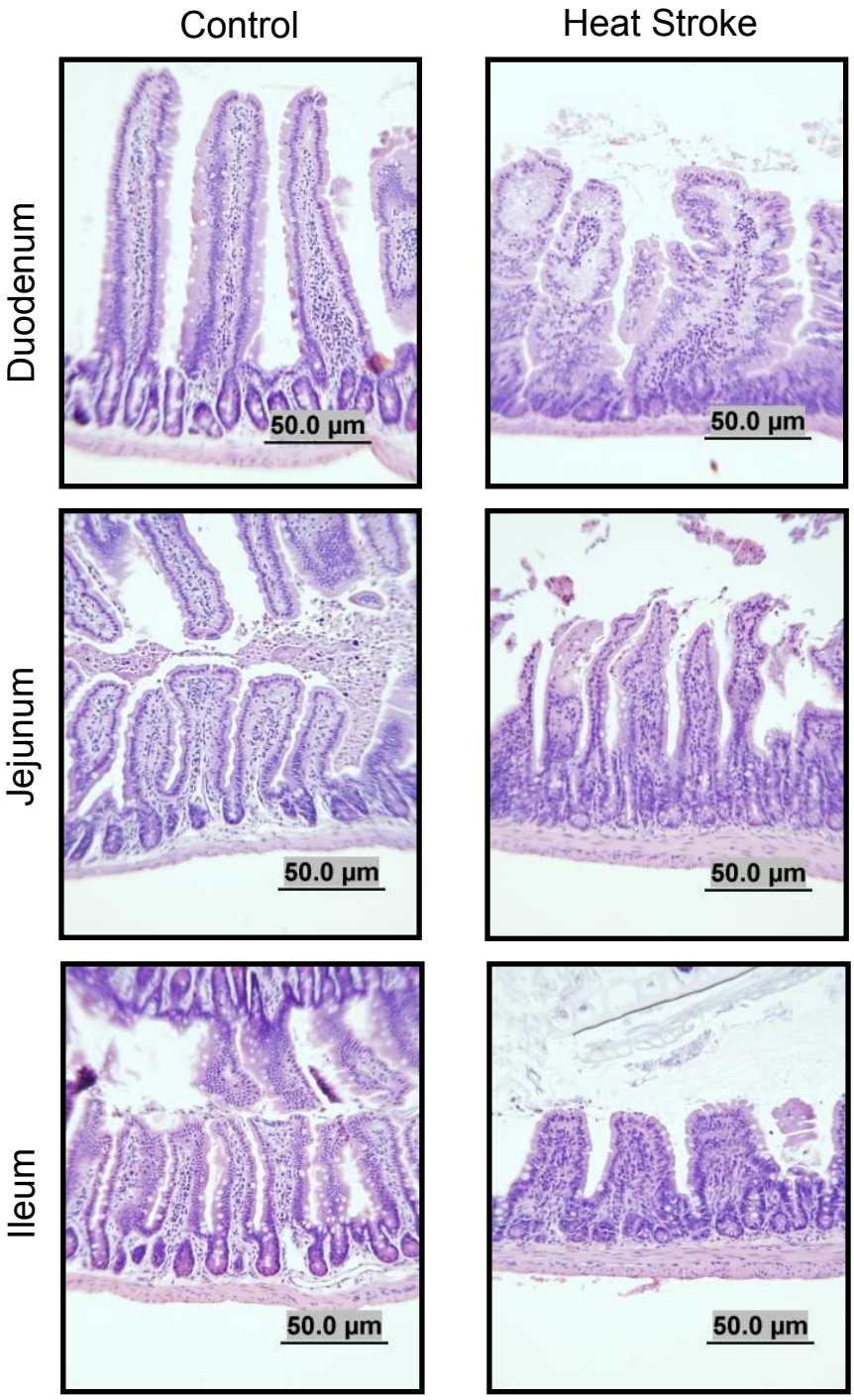

Figure 4-3. Representative histology by region and treatment. Note the epithelial lifting in HS duodenum and HS jejunum and increased swelling in HS duodenum.

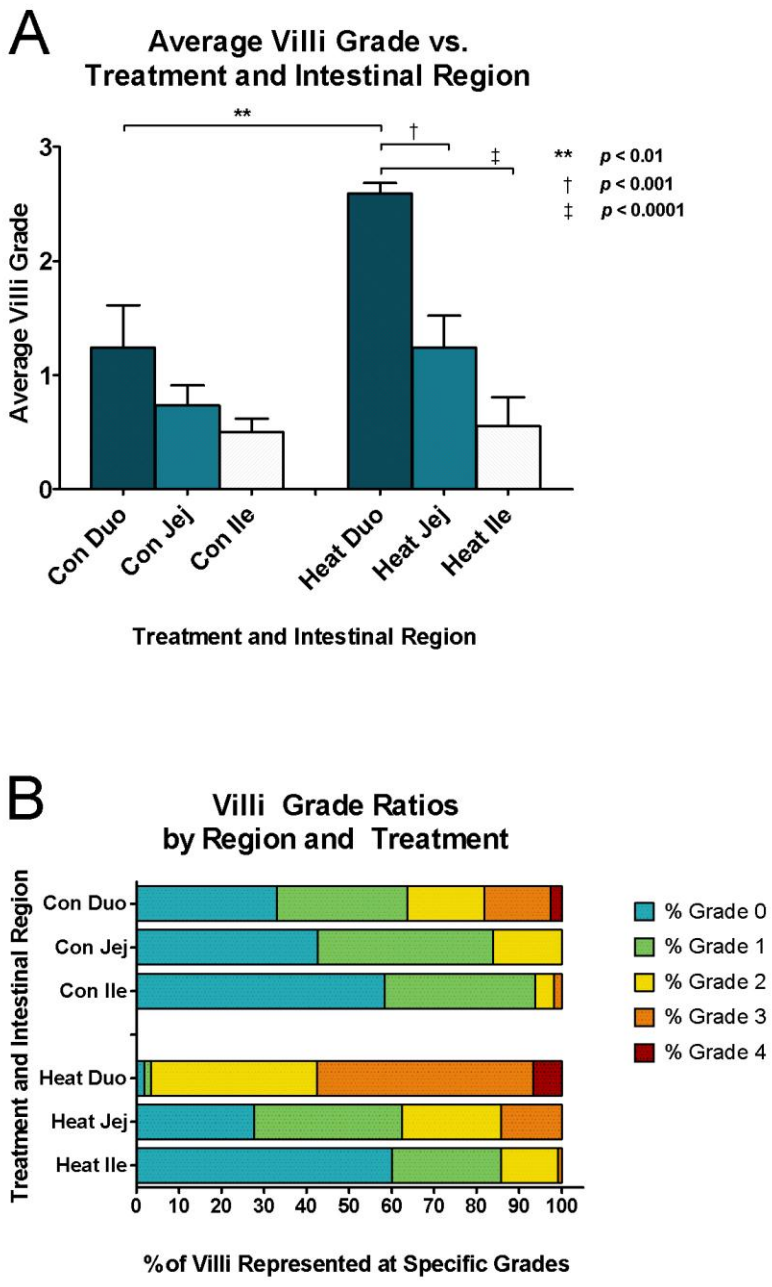

Figure 4-4. Villi grade averages and frequencies vs. treatment and intestinal region. A) Average villi grade vs. treatment and intestinal region: Heated duodenum showed significantly greater levels of damage compared to all treatments **($p < 0.01$) significant decrease in average damage score compared to heated duodenum. †($p < 0.001$) significant decrease in average damage score compared to heated duodenum. ‡($p < 0.001$) significant decrease in average damage score compared to heated duodenum. B) Villi Grade Ratios by Region and Treatment: The same data is broken down to display the frequency of specific grades.

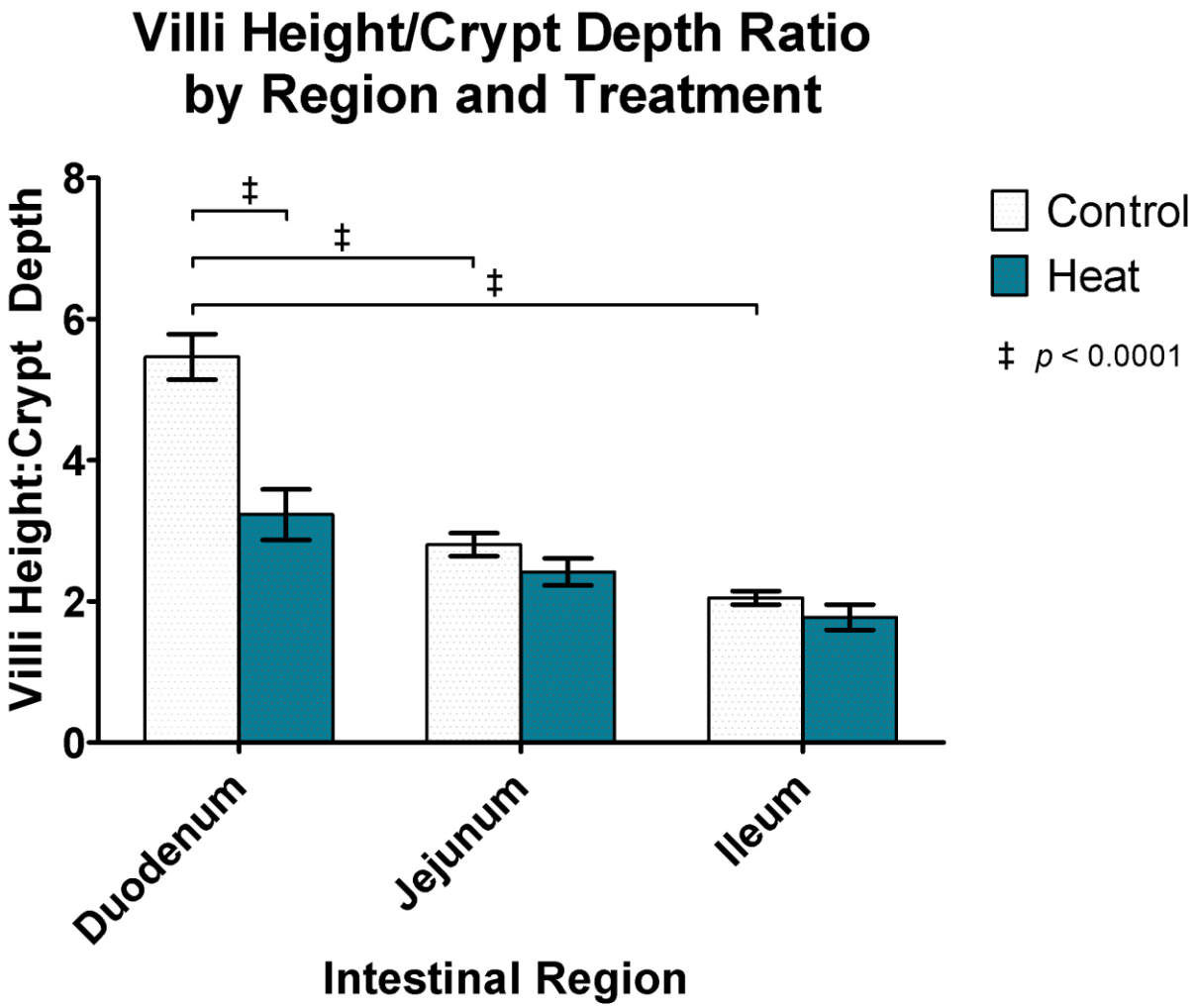

Figure 4-5. Villi height/Crypt depth ratio by region and treatment. ‡Control duodenum had significantly higher VH:CD over all other treatments ($p < 0.0001$).

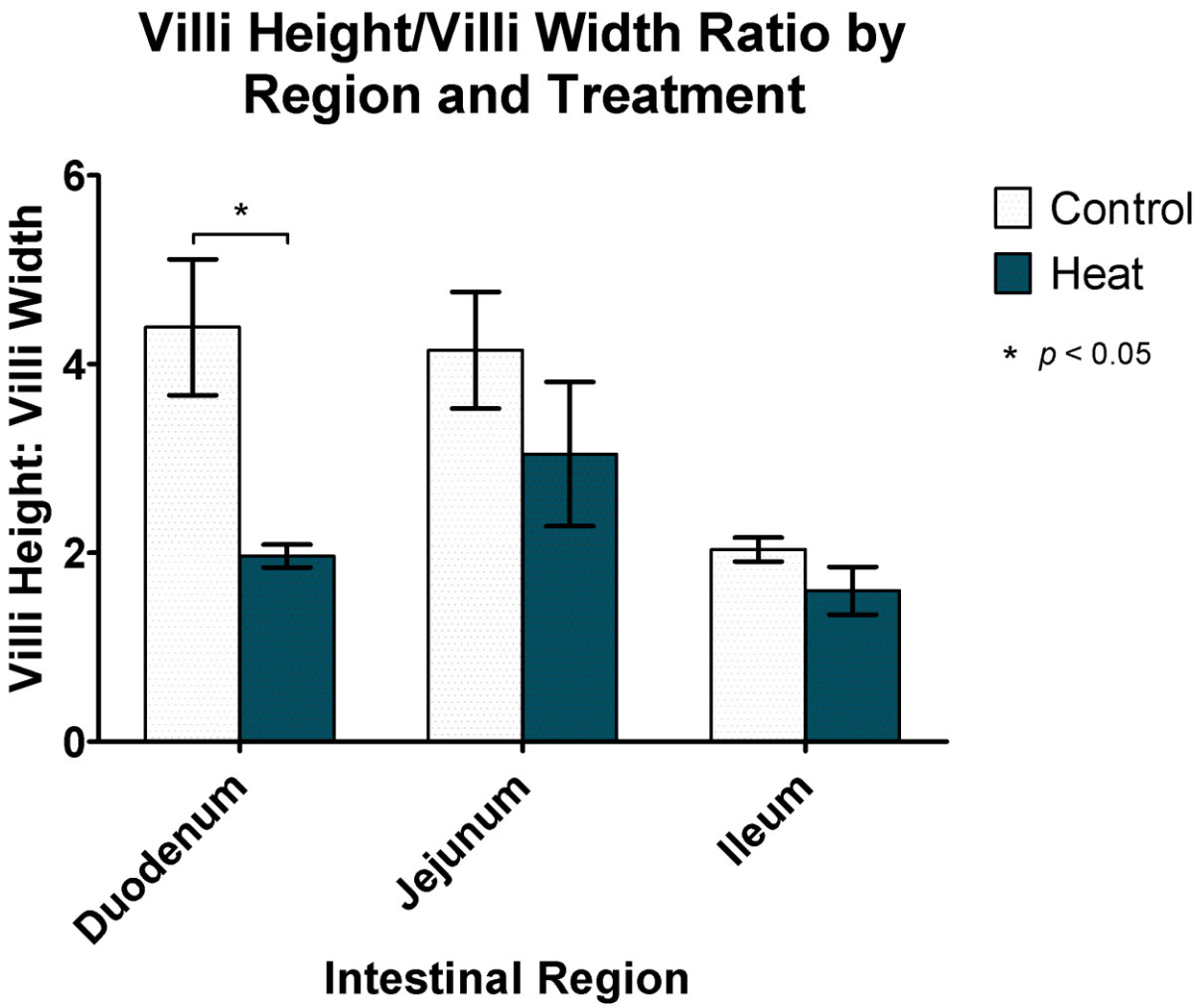

Figure 4-6. Villi height/Villi width ratio by region and treatment. *Control duodenum exhibited significantly higher villi height/villi width ratios than heated duodenum ($p < 0.05$).

CHAPTER 5
DISCUSSION

Summary of Results

Our evidence shows that simulated ischemia in an *in vitro* model and heat stress in an intact model of heat stroke results in a regional gradient in intestinal damage. In each stressor tested, *in vitro* ischemia, *in vitro* heat (previous work not part of this thesis), and *in vivo* heat stroke, we observed that the more proximal a region is to the stomach, the more susceptible this region was to damage. This gradient of injury appeared in both *in vitro* heat and ischemia as well as an *in vivo* model of heat stroke. More research is needed to determine if ischemia and heat have similar mechanisms of injury and/or if duodenum morphology is more susceptible to injury regardless of the stressor. Further explored, this knowledge could lead to regionally targeted therapies that increase intestinal barrier function in certain pathological or stress conditions.

Experimental Critique

In vitro and *in vivo* models were used to gain a more complete view of the intestinal response to heat and ischemic stress. Our *in vitro* model measured regional permeability and we originally tried to evaluate these *in vitro* segments for regional damage. However, the procedures left tissues with extensive apparent damage, making them of questionable value for histolopathology evaluation. Previously we had adjusted our *in vitro* methods to reduce variability; however, we suspected that after the tissue underwent standardized post-experimental measurements, it would be exposed to additional stress, which we believe is responsible for making the the tissues unsuitable for microscopic evaluation. When developing a model to test heat stroke *in vivo*, we considered dividing the small intestine with sutures or clamps and using

multiple colored probes to examine permeability. However, additional tampering could result in additional injury and/or altered blood flow. Thus to examine damage, we modified existing *in vivo* models to develop an approach minimizes invasiveness and keeps experimental conditions as physiologically relevant as possible.

There are multiple scoring systems to identify intestinal damage. However, many of these scoring systems fail to differentiate between potentially unique types of damage. Therefore, we chose to examine denudation, atrophy, edema, and intestinal permeability separately, with each having its own damage scoring system. To determine denudation and ultimately ulceration, we used the intestinal damage scoring system outlined by Chui et al. because it is widely used and its grades correlate proportionally to the extent and duration of ischemia (51). Our team modified the method by applying the grading system individually to a random sample of villi. By using the average grade of 10 villi, slides could be differentiated with greater accuracy and the frequencies of specific stages of denudation, could be determined. Villi height to crypt depth ratios have been previously used to objectively measure villous atrophy in intestinal barrier dysfunction in a variety of settings (52-58) and have been shown to result from cessation of blood flow (58). In the same fashion, villi height to villi width ratios were used to determine the extent of swelling (52). Finally, intestinal permeability was tested by measuring FD4 translocation. Together, this allowed the most complete picture using the best methods available to our lab.

Comparison to Previous Results

As previously mentioned in Chapter 2, at physiologic temperatures, regional permeability studies are conflicting, with different outcomes between species (42-44). A recent study, examining chronic heat exposure in pigs observed no VH:CD changes in

chronic heat exposure across duodenum, jejunum, and ileum (59). However, it should be noted that heat stroke was not induced in these animals. In acute heat stress, we observed a regional effect of permeability in the intestine while Lambert observed no significant effects (6). A weakness in Lambert's work was that too few microscopy samples were taken to determine the extent of mucosal damage; only three heat stressed mice, and one control mouse was used (6). We have effectively shown regional permeability in the duodenum *in vitro* as well as regional damage in the duodenum *in vivo.* It is possible that differences observed in the rat and mouse can be attributed to species differences; however, insufficient data is available in the rat model or in other mammalian species to make a definitive statement at this time.

Potential Causes of Duodenal Damage

Our results showed significantly greater damage in the duodenum compared to ileum, in both heated and control treatments. The endotoxin translocation theory states that the inflammatory cascade occurring in heat stress is a response to endotoxin seeping out of the gut (36, 60). It is hypothesized that endotoxin escapes the gut through openings in the tight junction of the epithelium, where it is transported to the rest of the body through the lymphatic system (36, 60). While this experiment was not equipped to view tight junction status specifically, we have observed that in heat stressed duodenal villi, over 50% of the villi sampled exhibited massive epithelial lifting down their sides and ulcerated, broken tips. In comparison, the jejunum exhibited less than 15% of sample villi to be ulcerated, and the ileum exhibited less than 1%. Because the majority of the damage was represented in the duodenum, one could assume that the duodenum may play a major role in the increased intestinal permeability observed in heat stroke.

The endotoxin translocation theory states that the inflammatory cascade seen in multi-organ failure is promoted mainly by endotoxin. However, the majority of bacteria inhabit the ileum and colon (61), which in our hands are not greatly affected by hyperthermia or ischemia. Few species of bacteria can survive or thrive in the duodenum due to its hostile luminal conditions and phasic propulsive motor activity (61). Therefore, our results might be more consistent to the transduction of some other inflammatory mediator than endotoxin (62). Additionally, the evidence regarding whether endotoxin is the main inflammatory mediator in multi-organ failure remains inconclusive, though it is generally accepted in the heat stroke literature (63).

The Auto-digestion Theory

The Auto-digestion Theory states that digestive enzymes such as proteases, lipases, nucleases, and amylases can also promote inflammation, upon leakage from the intestinal lumen (62, 64). The pancreatic duct regularly empties digestive enzymes into the duodenum where they are activated (62, 64). Normally, these volatile digestive enzymes are compartmentalized from the villi by a layer of mucous that covers the brush border and is impermeable to these enzymes (11, 62). In ischemic conditions, the mucous layer is damaged and becomes permeable to pancreatic enzymes, leaving the intestinal wall vulnerable to enzymatic penetration (62, 65). Tissues are digested and inflammatory mediators are produced from the degraded cellular components (62, 64). Exposure to pancreatic enzymes alone does not promote lukeocyte activation; however, the homogenate of tissues exposed to pancreatic enzymes will promote activation (66). In previous studies of ischemic intestinal injury, neither endotoxin nor TNF-α were present at detectible levels in the samples tested. Experiments were one third of the supernatant of pancreatic homogenate produced from one rat was placed

into a second, healthy, anesthetized rat resulted in 100% death in minutes (67). Further experiments showed that if the intestine is flushed with saline and the protease and phospholipase inhibitor, ANGD (6-amidino-2-naphtyl *p*-guanidinobenzoate dimethanesulfate, *nafamostat mesilate*), is administered, activation of circulating leukocytes will be almost completely inhibited in ischemia reperfusion injury (68). Together this suggests that pancreatic enzymes create distinct inflammatory mediators and that these inflammatory mediators can induce an inflammatory cascade without the presence of endotoxin. In addition, enzymatic digestion of intestinal tissues could be partially responsible for the villi ulceration and disintegration of the lamina propria seen in some specimens of duodenum.

Morphology of the Duodenum and Damage Susceptibility

Despite duodenum's proximity to the pancreatic duct, there are substantial levels of pancreatic enzymes throughout the small intestine. However, the duodenal barrier is less equipped to prevent these enzymes from coming into contact with the brush border. Research has shown that rat duodenum has a lower percentage of mucous producing goblet cells than other regions, starting at 4% at the duodenum and gradually increasing to 16% at the distal colon (69). The thickness of the total rat duodenum mucous layer is 170 µm, while the ileum is 476 µm (70). When this mucosal layer is removed by suction, the ileum replaces mucous at a faster rate compared to duodenum as well (71). With a decreased thickness and reduced restoration rate of mucous, the duodenum faces disadvantages to buffer itself against luminal contents. Duodenal villi are also typically longer than jejunal or ileal villi (71-73). The increased length may contribute to a greater oxygen disparity at the tip of the duodenal villi, making it more susceptible to ischemia (74) and ultimately mucosal damage (62). A longer villus will

also have increased surface area that is available to pancreatic enzyme inflitration. We theorize that the increased susceptiblity to damage in the duodenum may result from this unique combination of increased proximity to digestive enzymes, a thinner mucosal barrier with reduced regenerative properties, as well as an increased ischemic risk factor.

Further Applications

Severe cases of exertional hyperthermia are associated with GI dysfunction and endotoxemia (75-77), particularly in extended endurance events such as marathons (78) or triathlons (79). Recent findings suggest that 25-50% of elite athletes are afflicted with gastrointestinal symptoms that may affect their training (80). It is theorized that this exercise induced GI dysfunction is brought on by ischemia and increased intestinal permeability as exercise further reduces splanchnic blood flow when blood is shunted to the muscles (4, 80). More research is needed to determine if to what role and to what extent duodenal damage plays in these exercise induced gastrointestinal disturbances.

Summary of Conclusions

In conclusion, our results show that the duodenum is regionally susceptible to heat stroke in mice during and after exposure to extreme hyperthermia consistent with temperatures experienced in heat stroke in humans. This trend is exhibited both in an intestinal segment model and in anesthetized mice. More research is needed to determine to what extent the duodenum plays a role in non-exertional and exertional heat stroke. In addition, work is needed to determine if a heat stroke damaged duodenum compromises exercise performance.

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BIOGRAPHICAL SKETCH

Veronica Lea Novosad completed her applied physiology and kinesiology undergraduate degree at the University of Florida, specializing in exercise physiology. After graduating with her Bachelor of Science in August 2009, she stayed at the University of Florida and immediately enrolled in a master's program for applied physiology and kinesiology. She has since worked as a research assistant under Dr. Clanton. Within the Clanton lab, Veronica developed a simulated ischemia model, examined intestinal integrity in heat stroke, has contributed to four abstracts, and personally presented two of them at the international 2010 and 2011 Experimental Biology Meetings. She has currently been accepted into several medical schools and plans to attend in the Fall of 2011.