SMALL BOWEL SUSCEPTIBILITY TO HYPERTHERMIA: DEVELOPMENT OF A MODEL

By

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To my Mom

ACKNOWLEDGMENTS

Thanks, to those with the time to administer words of encouragement; to my family and coaches.

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LIST OF ABBREVIATIONS

FD4	Fluorescein isothiocyanate (FITC)-Dextran 4000 Da; a fluorescent protein sized to mimic small endotoxin (LPS) and is used to measure tissue permeability.
LPS	Lipopolysaccharide, or endotoxin; bacterial components in the intestine that stimulate immune and inflammatory responses
NSAID	Non-Steroidal Anti-Inflammatory Drug

Abstract of Thesis Presented to the Graduate School
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Our goal was to develop a viable model of heat-induced intestinal barrier dysfunction. Additionally, we tested the hypothesis that the small intestine displays varied changes in hyperthermia-induced permeability along its length. We isolated and everted segments from the small intestine of adult mice, creating ~8 x 2 cm sacs from each mouse, which were then separated into heated and control groups (42 $\pm$ 0.5^{o}C and 37 $\pm$ 0.5^{o}C, respectively). Permeability was measured following a 90 minute exposure. The data show a significantly increased permeability in regions of the intestine closest to the stomach (P ~ 0.009). We conclude that in the mouse, the anterior portions of the small intestine (near the stomach) have an increased susceptibility to hyperthermia compared to posterior regions (the ileum). This is of importance to our laboratory, as we have identified a key variable in evaluating permeability responses seen in our model. Our data further suggest that treatments targeted to protect the upper intestine may be useful in reducing the symptoms of heat related illnesses.

CHAPTER 1
INTRODUCTION

The small bowel is a critical site for the body's interaction with the external environment. The majority of nutrient absorption occurs across the epithelial wall, which serves as an anatomical barrier to anything ingested. As with most mucosal linings, the small intestine is home to a host of secretory, immune, and inflammatory cells that regulate the organism's interactions with dietary and bacterial antigens (Baumgart, 2002). The small intestine coordinates one of the body's largest collections of endocrine and immune cell populations while maintaining a symbiotic relationship with the resident bacterial milieu (Merchant, 2007). This relationship, hinged on proper barrier maintenance, is pivotal for immunological homeostasis.

The outermost layer of the intestine consists of a longitudinal and subjacent circular muscle layer. These muscles produce the peristaltic movements of the small intestine and control, downstream of neural stimulation, the motility of intestinal contents. The submucosa that follows this outer muscular layer is separated from the inner mucosa or lamina propria by the thin muscularis mucosae. The lamina propria is home to a variety of immune and inflammatory factors and is generally convoluted into finger-like villi that increase the absorptive area of the small bowel. Each villus has its own circulatory supply and is surrounded by invaginations known as "crypts". The lamina propria is then separated from the lumen by a unicellular layer of epithelial and differentiated cells. The luminal surface of these cells is impermeable to most hydrophilic solutes, a property accomplished through mucus secretion and strong intercellular connections (Turner, 2009).

Solute Movement

Solute movement across the intestinal barrier occurs either through paracellular (between cells) or transcellular (across cell membranes) pathways. Molecules too large or hydrophilic must rely on transporters on the apical surface of epithelial cells to access the body, often taking advantage of a stringent sodium gradient maintained by sodium-hydrogen co-transporters and intercellular tight junctions (Baumgart, 2002). Through cytoskeletal connections and intracellular signals, interactions at the apical surface can directly affect the assembly of the tight junction proteins that control paracellular solute diffusion (Turner, 2009). Cellular and tight junction integrity must be maintained to prevent the unregulated translocation of luminal contents, including bacteria and bacterial products (endotoxin), into the submucosa.

To assist in its immunological role, the small intestine hosts a large number of B cells, T cells, macrophages, and other lymphoid tissue (Baumgart, 2002). Antigen presenting cells (APCs), like dendtritic cells, and pattern recognition cells, like the toll-like receptor (TLR) family reside in the lamina propria and link the body's innate and adaptive immune responses. These components interact with specific stimuli and guide the body's adaptive responses by activating resident B and T Cells. Toll-like receptor-4 (TLR-4), a member of the toll-like receptor family that is sensitive to LPS in the intestine can induce inflammation by stimulating the local production of cytokines by lymphocytes. Intraepithelial lymphocytes are joined at the mucosal surface by differentiated epithelial cells, including the absorptive brush border cells and mucus-secreting goblet cells that are constantly generated from crypt stem cells and develop along the length of the villus (Turner, 2009).

Mucus Layer

The first line of defense for the epithelium against fully active digestive enzymes and bacterial antigens is a continuous, secreted mucus-bicarbonate layer (Allen, 1986). There are actually two distinct layers, a loosely- and a firmly-adherent mucus layer, both formed by epithelial secretions of mucin, bicarbonate, and a glycocalyx, or sugary coat. The loosely adherent layer plays a superficial protective role and is more susceptible to damage by ethanol, aspirin, and high concentrations of pepsin and bile salts (Allen, 1986). The firmly adherent layer is vital in protecting mucosal cells from luminal acids and for maintaining a relatively neutral juxtamucosal pH along the length of the intestine; its thickness varies along the gastrointestinal tract and is lowest in the small intestine (Atuma, 2001). In addition to its defensive role in protection from intestinal contents, an undisrupted mucus layer improves intestinal function. It limits diffusion, aiding nutrient absorption and immune function by controlling the rate at which nutrients and antigens come into contact with the mucosal barrier. By also allowing the concentration of epithelial secretions to remain high, it increases the activity of locally produced digestive enzymes, and makes bicarbonate secretion more effective at maintaining a neutral pH at the luminal surface (Allen, 2005).

Tight Junctions

Interactions at the mucosal surface determine epithelial cell viability and regulate the assembly and disassembly of tight junction proteins. These intercellular proteins help regulate the flux of water into the central cavity of the small intestine, or lumen, and preserve the continuity of the intestinal barrier while controlling the most dynamic and permeable path of solute movement (Steed, 2010). At tight junctions, the space between cells is essentially eliminated, creating a physical barrier to luminal contents.

Moreover, the structural and regulatory proteins of the tight junction help establish the charge and size selectivity needed for the tightly controlled flow of solutes across the intestinal wall. Claudins are one such family of proteins known to help govern the cation specificity epithelial linings throughout the body (Niessen, 2007).

The dynamics of the tight junction can be regulated by the direct modulation of morphological structures or by the activity of effector proteins and molecules (Steed, 2010). Other junctional proteins include the intercellular proteins cadherin and occludin, and the scaffolding protein, zonula occludens 1 (ZO-1). Extracellular activation of cadherins occurs via calcium signals through the paracellular space; this increases junctional tightness and triggers intracellular signals to increase the transcription of junctional strands (Turner, 2009). Tight junctions are normally regulated by perijunctional rings of actin and myosin that are under the direct influence of myosin light chain kinase (MLCK). MLCK phosphorylates myosin II regulatory light chain (MLC) within the acto-myosin ring, activating myosin ATPase and stimulating interactions between actin and myosin. The resulting condensation of the perijunctional ring pulls on junctional proteins, controlling the strength of the tight junction (Turner, 2009). MLCK is a common endpoint of physiological and pathological mechanisms regulating intestinal permeability (Turner 2009).

Medical Interest

Myriad disease states and disorders trace their effects to intestinal dysfunction, including ulcerative colitis, inflammatory bowel disease, and heat stroke (Cario, 2000). Barrier maintenance is regularly tested as any number of pharmacological, psychological, physiological, or pathological factors can affect mucus secretion or increase intestinal permeability (Lambert, 2009). Proper barrier function involves the

establishment and maintenance of a sodium gradient across the mucosal membrane. Tight junctions help prevent the dissipation of this gradient by guarding solute movement between epithelial cells; alterations in these mechanisms can result in diarrhea from increased water flux into the lumen or deficient absorption (Turner, 2009). Damage to the cell membrane, a direct effect of increased core temperature, challenges the body's ability to maintain transmucosal gradients.

Patients with Crohn's disease and ulcerative colitis both possess upregulated TLR-4, which is primarily activated by endotoxin in the intestine and results in the overstimulation of T Cells (Cario, 2000). The decreased barrier function in colitis patients has also been attributed to an overexpression of the tight junction protein claudin (Steed, 2010). The expression of this protein varies between organs and throughout development and may be modified by cytokines like interferon-γ (IFNγ) and tumor necrosis factor (TNF) (Turner, 2009). Increased mucosal TNF production has been linked to a variety of intestinal disease states, including colitis, and has been shown to regulate tight junctions *in vivo* and *in vitro* by manipulating MLCK's enzymatic activity and by increasing MLCK transcription and translation. Because of its ability to alter mucus production, increase mucosal permeability, and cause malabsorption, TNF is often used as an experimental stimulus for intestinal dysfunction (Turner, 2009).

Heat Stress

Increases in core temperature, from the environment or exercise, can cause as much as a 40% reduction in intestinal blood flow (Hall, 2001) as the body attempts to simultaneously power active muscles and disperse heat by diverting blood to the skin. As a result, the intestine may become deficient in blood flow. This, when combined with direct thermal damage to membrane cells, disrupts tight junction proteins, allowing

bacterial components like endotoxin (Hall, 2001) and digestive enzymes to penetrate the mucosal barrier (Schmid-Schonbein, 2008). The subsequent rise in bacterial translocation activates both local and systemic inflammatory responses by accessing the circulation and infiltrating the liver and other organs. With increasing severity, the resulting endotoxemia and inflammatory cascades can result in sepsis or death (Hall, 2001).

A five hour heat exposure for three days notably decreased the integrity of tight junctions *in vivo* and increased in the number of damaged mitochondria in the epithelium (Liu, 2009). The internal cristae of mitochondria appeared swollen and shortened and there was an increase in secondary lysososomes; both are thought to occur in response to intestinal lesions caused by hyperthermia (Liu, 2009). Villus heights and crypt depths were significantly decreased in response to heat treatment and the desquamation of epithelial cells at villus tips and exposed lamina propria offered clear signs of mucosal damage (Yu, 2010).

Lambert et al, (Lambert, 2002) also reported thermal disruption of the epithelial membrane both *in vivo* and *in vitro* with physiological increases in temperature. The elevation of tumor necrosis factor TNF-α, a primary mediator of endotoxic shock, in heat stroke patients suggests a link between heat stress and entotoxemia (Lambert, 2002). Circulating levels of endotoxin (LPS) are also significantly increased in heat stroke victims and heat-treated animals (Hall, 2001). Dokladny et al (Dokladny, 2005) found similar effects in caco-2 monolayers, noting that increases in temperature decreased transepithelial electrical resistance (TEER) and increased the permeation of inunlin (a paracellular permeability probe) indicating a breakdown of tight junction stability.

Current Methods for Investigating Intestinal Permeability

There are various *in vivo*, *ex vivo*, and *in vitro* animal models that have been verified for the investigation of intestinal function, allowing investigators the freedom to manipulate specific physiological parameters. Recently, the effects of long term heat exposure was investigated by subjecting experimental mini-pigs to a heat stress for five hours daily for 10 consecutive days. Animals were sacrificed at different time points and epithelial tissues were excised for morphological analysis using hematoxylin and eosin (H&E) staining and transmission electron microscopy to visualize nuclei and cellular components. The relative lengths of villi and crypts were recorded and used in conjunction with photomicrographs to quantify damage to the epithelium (Yu, 2010). After three days of treatment, thermal damage was noted in jejunal epithelium by shorter villus heights and crypt depths; alterations in tight junction morphology were determined by localized changes in electron density (Liu, 2009).

A common method of assessing intestinal permeability, both *in vivo* and *in vitro*, uses the ratiometric passage of an ingested pair of hydrophilic permeability probes into the urine or blood. Probe pairs typically consist of a large and a small saccharide that are selected based on established regions of permeation along the digestive tract; the sugars lactulose and mannitol are often used to assess the permeability of the small bowel (Fink, 2003). These probes have been used to assess the *in vivo* human permeability of gastric or intestinal mucosa, but their use relies on several critical assumptions. First, that the larger sugar (lactulose) is limited to paracellular diffusion while the smaller (mannitol) can diffuse both between cells and across the apical cell membrane. This method does not account for the possibility of active transport, and the rate of absorption of the smaller probe is presumed to be constant. Moreover, both

sugars are assumed to be equally affected by changes in intestinal permeability or alterations in pre- and post-intestinal factors like transit time or renal function. The assumptions of this model have come under question with the temperature-dependent active transport of a variety of hydrophilic compounds, including lactulose, being verified in the small bowel (Tomita, 2000).

Barrier function is investigated by analyzing the status and function of the protective mucus layer. As it may be easily disrupted during *in vitro* and staining procedures, changes in the structure of the adherent mucus layers are optimally studied *in vivo*. Intravital microscopy is used to measure mucus thickness and production in the exteriorized and mounted gastrointestinal tissue of anesthetized rats (Atuma, 2001). Szabo et al, (Szabo, 2005) used an intravital microscopic method to measure the accumulation of small and medium-sized fluorescent markers in the intestinal interstitium *in vivo* following an ischemic event. This method provides a relatively easy and applicable means of assessing epithelial permeability *in vivo* and of evaluating the efficacy of attempts to preserve barrier function. However, it does not lend itself to quantitative assessment between treatments.

The use of cell culture systems is another option. Though their use diverges from normal physiology, it has the advantage of being highly controllable. In addition, the method sometimes yields results that are variable and sometimes of questionable physiological significance. Caco-2 cells are a line of colonic cancer cells that are often grown into monolayers and used in intestinal permeability studies. The effects of temperature on Caco-2 permeability can be determined using established paracellular markers like inulin (Ma, 1991). Electrodes placed on either side of the monolayer

measure are often used to measure transepithelial electrical resistance (TEER) as an additional assessment of solute movement between cells (Dokladny, 2005). Properly functioning tight junctions must establish and maintain electrical resistance across the epithelium and changes in TEER may represent early signs of cellular damage. However, there are normal openings in tight junctions that are responsible for ionic and water movements that do not represent the same cytoskelelal motions responsible for barrier dysfunction. The measurements of electrical resistance are therefore not suitable for determining the mechanisms responsible for diffusion of large molecular weight solutes like endotoxin.

Combining the techniques of time-lapse multidimensional fluorescence microscopy and measuring (TEER), Turner et al, (Turner, 2009) visualized the organization of fluorescent-tagged tight junction proteins. This methodology is used to examine the assembly and dissociation of these proteins before, during, and following experimental conditions. Similarly, the immunostaining of Caco-2 monolayers has also been used to visualize the behavior of junction proteins and their cytoskeletal connections in response to increases in temperature (Dokladny, 2005).

The use of Ussing diffusion chambers allows tissue from different regions or species to be treated similarly for the comparison of direction- and condition-specific solute movement and analysis (Nejdfors, 2000). Simultaneously, TEER can be measured across the tissue as an indication of cellular viability. Specifically sized molecular weight markers are used to examine the different pathways of solute permeation, and proper selection can offer a complete picture of barrier integrity. Larger markers like α-lactalbumin and ovalbumin are used to show mucosal

permeability to macromolecules. Medium sized molecules, like the 4 kDa fluorescein isothiocyanate (FITC)-dextran (FD4) or radiolabeled ethylenediaminetetraacetic acid (EDTA; an organic compound), are known to permeate the barrier using mainly the paracellular route in the absence of overt cellular damage (Nejdfors, 2000).

In one such experiment, biopsies of the gastrointestinal tract were obtained from patients undergoing colonic resections and malignancy removals in the stomach, esophagus, or colon (Ungell, 1997). After the external muscle layers are stripped, the movement of radiolabeled mannitol, ovalbumin, alpha-lactalbmin, FD4, and FD70 (FITC-dextran, 70 kDa) across the epithelial barrier was measured in an Ussing chamber while electrodes measured TEER. By employing compounds known to use each avenue of solute permeability, investigators overcame the limitations of ratiometric sugar and protein calculations.

Lambert et al, (Lambert, 2002) investigated the role of oxidative and nitrosative stress in hyperthermia-induced intestinal permeability, using an *in vitro* segmental model in rats. After excision, the small intestine was cleaned, everted, and separated into individual, buffer-filled segments that were then exposed to temperature or compound specific treatments in a solution containing a medium or large fluorescent marker (0.25 mM FD-4 or FD-10). The movement of the fluorescent markers, in conjunction with volume and area measurements, was used to calculate tissue permeability in response to heat treatment. Lambert et al's heat-induced permeability studies (Lambert, 2002) in rats utilized both *in vivo* and *in vitro* models and compared the results, finding that the segmental *in vitro* model used mimicked *in vivo* results (Lambert, 2002). This method permits the study of multiple conditions separately using

tissue from a single animal, while presumably obtaining physiologically relevant results. In addition, the method allows evaluation of the intestine, *ex vivo,* after a treatment is given to the intact animal. For this reason our laboratory chose to develop this model in the mouse in order to study the pathophysiology of heat stroke.

A previous student in our laboratory, S. Ryan Oliver, adapted Lambert's protocol for the development of our laboratory's own murine (mouse) model of heat-induced barrier dysfunction. This model was used to evaluate different treatments to the tissue that could attenuate the hyperthermia-induced permeability, but the level of variance amongst similarly treated tissues made it difficult to measure significant effects (Figure 1-1). Changes were made in tissue and buffer preparations in attempts to reduce this variability before the decision was made to average duplicate treatments within each animal. This compensated for the variance, but failed to sufficiently address the sources of variance. Their identification and elimination became the focus of my graduate work.

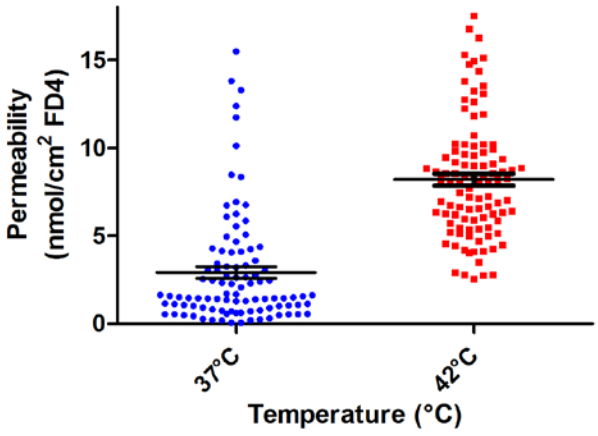

Figure 1-1. Variance in original protocol. N = 49 Mice

Specific Aims

The specific aims of the present investigation were to 1) identify and address the sources of variability in our *in vitro* segmental model of hyperthermia-induced intestinal permeability, 2) to determine the relationship between intestinal region and heat-induced permeability, and 3) to test the hypothesis that intestinal permeability varies as function of proximity to the stomach.

CHAPTER 2
METHODS

Chemicals Used

Medium 199 (Cellgro), L-Glutamine (Lonza), sodium bicarbonate (Acros Organics), fluorescein isothiocyanate (FITC)-dextran 4 kDa (FD4, Sigma Aldrich)

Animal Treatment and Gut Sac Preparation

Our studies used adult C57bl6 mice (25-35 g). Animals were treated according to protocols approved by the University of Florida Institutional Animal Care and Use Committee. Prior to euthanasia by carbon dioxide asphyxiation, mice were kept on a clear liquid diet for 10 hours to reduce luminal contents. The entire intestine was then rapidly excised and placed in pre-oxygenated medium 199 (with glutamine and sodium bicarbonate). Excisions were made proximal to the cardiac sphincter in the stomach and distally in the large intestine. After gross removal of the mesentery – responsible for vascularization – the small intestine is freed of the cecum and large intestine with a cut above the ileocecal valve. The remaining mesentery is then trimmed, taking care throughout not to damage the gut.

After the small intestine was made linear, its contents are flushed with oxygenated medium 199. Next, the length of the intestine is everted over a smooth glass pipette that has been gently inserted into the luminal cavity. The gut is then filled with oxygenated media and 1-2 cm sections are sequentially isolated using 2-0 suture. Next, gut sacs were placed into cuvettes of oxygenated buffer containing the high molecular weight fluorescent marker 4 kDa fluorescein isothiocyanate-dextran (0.3 mM FD4). The cuvettes were then covered and placed into water baths maintained at 37°C or appropriately set aluminum heating blocks.

The temperature was monitored with a highly accurate thermistor, accurate to two digits (Yellow Springs Model 4610). Heating blocks were controlled by a self regulating temperature controller (Digi Sense, Cole Parmer) and a heating plate. After experimental exposure, the solution inside the gut sacs were collected in individual tubes and the square area determined after longitudinal dissection. The FD4 concentration of the serosal fluid was calculated by comparing the fluorescence measurements made in a spectrofluorometer (SpectraMax M5, Molecular Devices) to those of a standard curve.

Throughout my investigation and attempts to lessen the variance of this model, several changes were made to our methodology. Food was no longer withdrawn from the animals prior to euthanasia and smaller glass pipettes were used for eversion. Prior to gut sac preparation, the intestine was filled under a defined column pressure of ~6 cm of oxygenated media. Segments were then prepared to a standardized length of ~2.3 cm. Randomized pairs of segments were then incubated at 37°C in conical tubes containing continually oxygenated buffer. Following this 30 minute period, segments were transferred to conical tubes containing 0.3 mM FD4 and placed into water baths maintained at 37°C or 42°C. Finally, after experimentation, tissue area was measured by compression with a standardized weight instead of segmental dissection.

Data Analysis and Statistics

At the conclusion of the experiment, sac contents were emptied into individual, pre-weighed tubes. The serosal volume of the sac was calculated from the change in weight and recorded in milliliters. In the original protocol, the segment was then dissected longitudinally and flattened to record the length and width. This was adapted so that the segment was compressed with a standardized weight and the square area

measured. The micromolar concentration of the serosal fluid was determined by comparing its fluorescence reading to that of a standard curve. Tissue permeability was normalized to segment surface area and serosal volume and was represented as the transport of nmoles of FITC-dextran per cm2. This analytical method was taken from Lambert et al. (Lambert et al., 2002) who denoted permeability = ($Concentration_{serosal\ fluid}$ X $Volume_{serosal\ fluid}$) ÷ Mucosal surface area.

Decreases in permeability measurements were determined using Analysis of Variance (ANOVA) or Student's t test for unequal variance (GraphPad Prism). Reductions in variance following adaptations were calculated from Fisher's F test for difference of variance (SASJMP). Multiple logistic regression analysis was used to stratify potential sources of variance (SASJMP). The slopes of baseline and heated treatments were analyzed using comparative linear regression to examine linear plots of data (GraphPad Prism). All results are reported as means ± SEM; $P < 0.05$ was considered to be statistically significant.

CHAPTER 3
RESULTS

Change in Experimental Chamber

The original protocol that was developed in the laboratory (Oliver, 2009 Thesis) used small 3 mL sealed cuvettes for each intestinal segment. Though this may have modeled the expected ischemic conditions of hyperthermia in the whole animal (Hall, 2001), I was concerned that variations in PO_2, PCO_2, pH and nutrient depletion within the cuvettes may have affected the permeability and been a source of variance. I therefore, first quantified the acidity and gas concentrations in the covered 3 mL cuvettes, using the original protocol in both heated and non-heated cuvettes. At experiment's end, the values for pH, PCO_2, and PO_2 were measured using an arterial blood gas analyzer (ABL800 FLEX) (Table 2-1). Because the blood gas machine brings all samples back to 37°C, samples taken from different temperature cuvettes are renormalized to the same temperature. While the mean for pH was consistent between baseline and heated treatments, individual values were lower than expected given the sodium bicarbonate buffer system we used. This was probably due to the lack of bubbling of the cuvettes during the treatment and the clear accumulation of PCO_2. The values for PO_2 were lower than expected. Combined with the acidity and hypercapnia noted in a number of treatments, this data verified our need for using a different system that controlled the gas concentrations as well as temperature throughout the protocols.

We addressed this need by replacing the sealed cuvettes with adapted fifty (50) ml conical tubes. These vessels allowed control over a steady, low bubbling of 95% O_2/5%CO_2 into the incubation and treatment media, which can be accessed and monitored without disrupting experimental conditions. As high rates of bubbling can

saturate the soluble CO_2 and lower the pH of the solution the bubbling rate was kept low. Using this method, permeability was measured over 90 minutes from segments treated with only 37°C or 42°C in conical tubes. These were compared with results from to similarly treated segments in the 3 ml cuvettes (Figure 2-1). With the change in vessel, we noted a decrease in the permeability ($P < 0.008$, t test for unequal variance) and more importantly, a significant reduction in the variance between the temperature treatments ($P < 0.05$, F test).

Area Measurements

Following the success of our vessel change, we questioned the accuracy of our method of estimating surface area of the segments after treatment. This calculation is needed to normalize the measurements to the surface area for diffusion. The original technique had a great deal of uncertainty in it and we wondered if it may cause additional tissue damage and distort the area measured. The original method called for a longitudinal dissection of each segment and before it was laid flat and its length and width recorded. To reduce the potential for variance, the decision was made to compress the tissue without cutting it, by laying it between two (2) small sheets of plastic with a standardized weight on top. We desired to select a range of weights that would sufficiently flatten the sample without causing tissue deformation and at which moderate increases in weight – from water tension or hand pressure – would not significantly alter the area measurement. To standardize this method, and select an ideal weight, we measured tissue compliance on eight segments following a ninety (90) minute 37°C treatment. The weights placed on the segment were progressively increased until a plateau was achieved. (Figure 2-2) A standardized weight of 30 grams was selected from the point of inflection on the compliance curve seen in plots of area

measurements against the corresponding weight applied. To examine the effect of this change on the variance and the value of the permeability measurements technique, we compared data from control samples that were first compressed vs. those that were split and dissected following 37°C or 42°C treatment (Figure 2-3). The means were compared with ANOVA and variances compared using Fisher's F test. Compression resulted in stark reductions both in the variability within temperature-specific groups and in permeability measurements ($P < 0.05$). The lowering of variance in both baseline and heated treatments indicates the elimination of another source of variance in our system allowing it to be more efficiently used to examine manipulations of barrier function.

Analysis of Confounding Variables

Despite successes improving the consistency of our model by altering our apparatus and by standardizing the area measurement, we were still faced with a level of variability that hindered our ability to assess treatment effects. A multiple regression analysis was then performed using essentially every different variable that we could measure in order to possibly reveal any strong experimental factors that would affect the variability of our outcomes (Table 2-2). The factors, area, fluorescence, serosal volume, and FITC-dextran concentration were expected to have major effects on the variance as they are each included in the calculations of permeability. However, the region of the small intestine from which the sac was made, denoted as "segment number" and later as a relative distance along the intestine, was found to be highly significant in affecting our variability ($P < 0.005$).

We then examined empirically the effect of segment number, which was previously treated as arbitrary. This was not originally thought to affect permeability as two different studies reported that it made no difference (Oliver, 2009; Lambert, 2002). To

more specifically record the tissue's location along the small intestine, the distances of calculated midpoints of each segment along its length were then used instead of arbitrary segment numbers. Sequential regions of the small bowels of four (4) adult mice were exposed to either 37° or 42°C in one of four (4) assigned conical tubes. The midpoint for each segment was plotted against the permeability value calculated from the volume, area, and fluorescent measurements made following their specific treatment. In the plot of these points, distinct lines can be fit to similar treated tissues (Figure 2-4). The slopes of these lines may then be statistically compared using linear regression analysis. At 37°C there is a proportional relationship between the proximity of the tissue sample to the stomach and its permeability. This indicates an increased susceptibility to epithelial damage in the duodenum and jejunum, the proximal small intestine, when compared to the more distal ileum. This relationship appeared stronger in heated tissues in the increased slope of the line for heated tissues, but failed to reach statistical significance.

Tissue Handling

In attempts to further reduce the variability in our intestinal experiments, I made a number of modifications in our tissue handling procedure. This came through working closely with another student, Veronica Novosad, in the laboratory to refine a standardized methodology for isolation. Efforts were made to limit mechanical stresses on the intestine during eversion and gut sac preparation. We ensured proper oxygenation throughout segment preparation, incubation, and treatment, and standardized the amount of buffer used to flush intestinal contents and the hydrostatic pressure on intestinal walls during gut sac preparation. Together, these methodological adaptations further lowered the variance of our data (Figure 2-5). This can be seen in

the increased accuracy of the lines associated with both baseline and heated groups. Gastric segments are more sensitive to increases in permeability with our improved methodology. This regional susceptibility is maintained at higher temperatures and appears to be strengthened with heating, though not sufficiently for statistical significance.

Table 2-1. Conditions in cuvettes. Values compiled from 3 Mice.

Treatment	pH	pCO2	pO2	Permeability
37°C	7.1	73	91	1.86
37°C	7.4	40	96	1.77
37°C	7.2	53	94	5.22
37°C	7.3	45	88	1.27
Mean	**7.3**	**53**	**92**	**2.53**
42°C	7.2	52	80	8.33
42°C	7.3	49	86	6.37
42°C	7.2	53	80	6.14
42°C	7.5	28	76	14.83
42°C	7.2	52	72	8.15
Mean	**7.3**	**47**	**79**	**8.76**

Heat treatment increases permeability ($P < 0.05$)

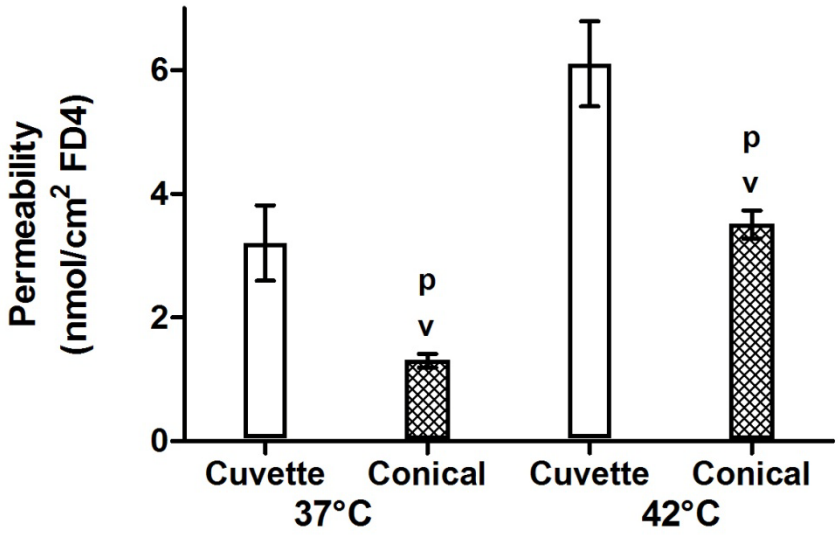

Figure 2-1. The Effect of Vessel on Permeability. (p) and (v) indicate that permeability and variance are lower in conical tubes than cuvettes N = 41 mice, P < 0.05

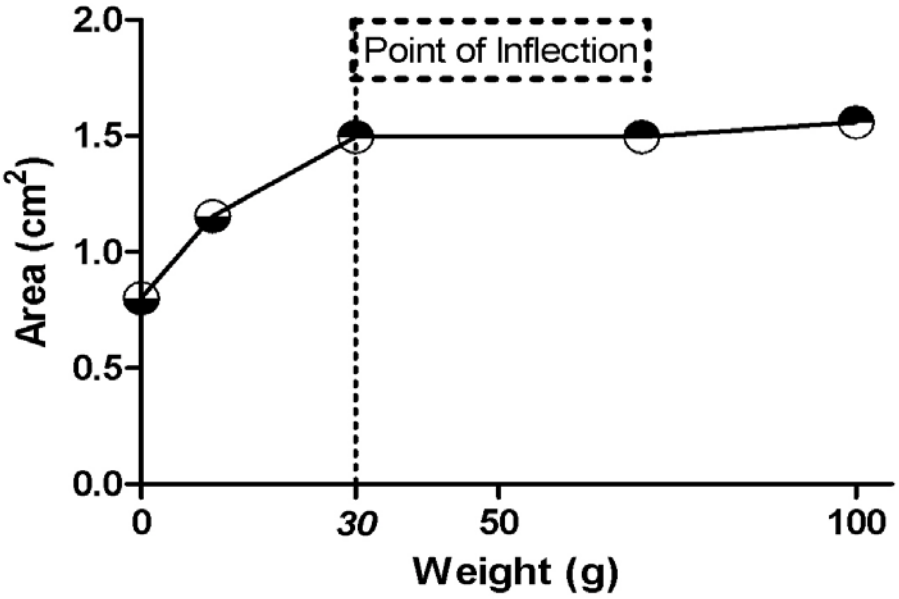

Figure 2-2. Mean Compliance Curve. Average inflection point at 30 ± 5 g. N = 8 sacs

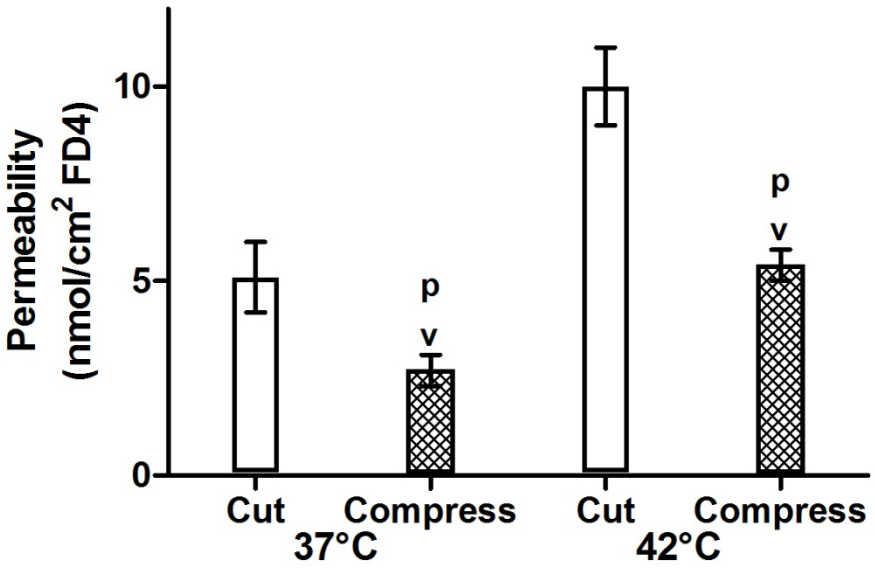

Figure 2-3. Change in Area Measurement. (p) and (v) indicate that permeability and variance are lower with compression than dissection. N = 15 mice, $P < 0.05$.

Table 2-2. Experimental sources affecting variance. A multiple logistic regression analysis

Technical Sources	**Significance (P-Value)**
FD4 Concentration	< 0.0005
Serosal Fluorescence	< 0.0005
Serosal Volume	~ 0.1
Tissue Area	< 0.05
Biological Sources	
Segment / Region	< 0.0005
Temperature	< 0.0005

FD4 Concentration: Serosal concentration of fluorescent probe
Serosal Fluorescence: Raw fluorescence reading of serosal volume
Serosal Volume: Fluid contents of intestinal segment
Tissue Area: Square area of intestinal segment
Segment: Anatomical region from which segment was prepared
Temperature: Temperature-specific experimental exposure

Statistical Significance at $P < 0.05$

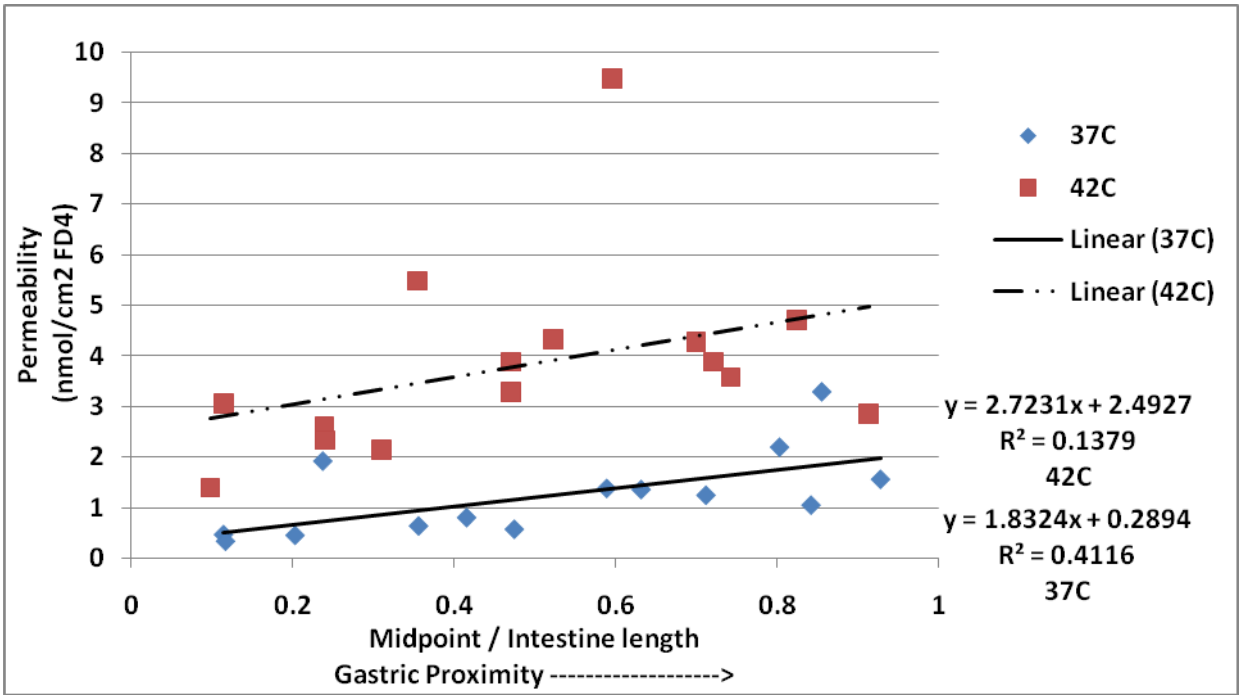

Figure 2-4. Regional permeability. Permeability increases as a function of gastric proximity. N = 4 mice

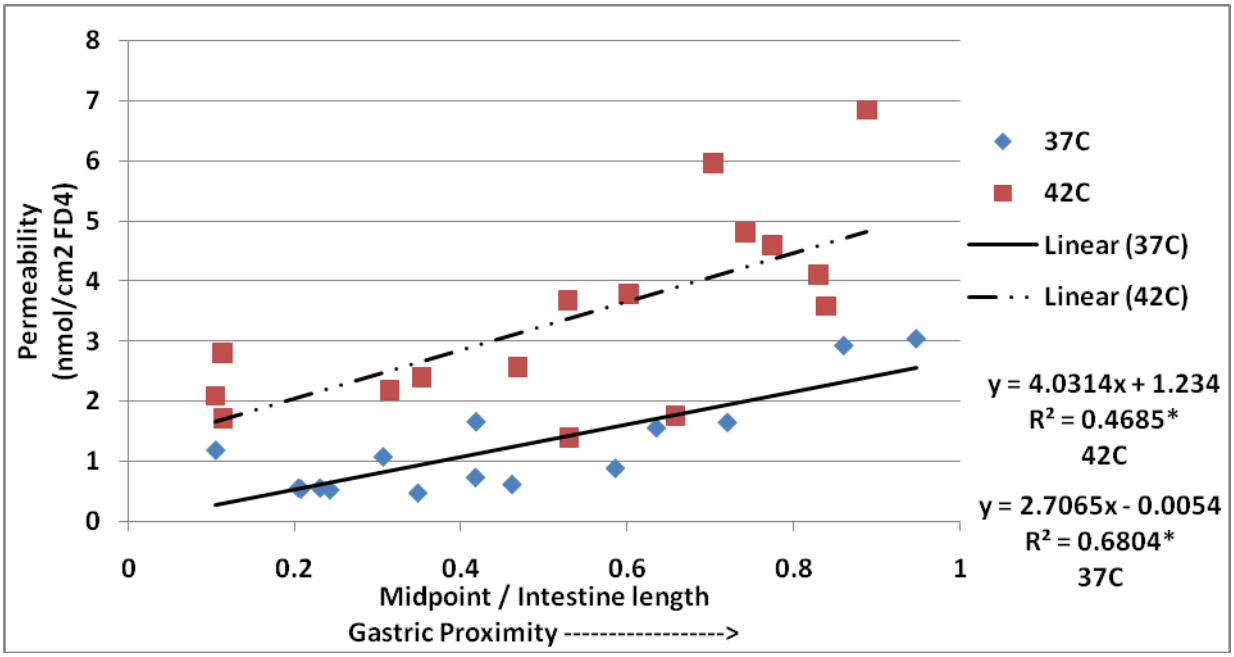

Figure 2-5. Improved tissue handling. Specific considerations to eliminate investigator-induced damage increased predictability (*) compared to Figure 2-4 and made more visible the influence of anatomical region. N = 4 mice

CHAPTER 4
DISCUSSION

At the outset, I was presented with a model having such a large degree of variance to it that the laboratory was unable to distinguish the effects of treatment over and above the inherent variability of the measurements. Through the systematic and rigorous deconstruction and analysis of our experimental model, I successfully resolved a number of technical issues that when understood and accounted for significantly improved the overall methodology and improved the resolution. My goal was to identify, explore, and resolve the sources of variance within our model, thus successfully increasing the predictability of control segments and allowing us to achieve full model viability. Experimental confounders may result from investigator manipulation or may be biological, resulting from the physiological and morphological nature of the intestinal mucosa. Surprisingly, based on the work of previous investigators, anatomical region of the small intestine, a biological variable, was paramount amongst these confounders. The susceptibility of gastric regions to heat-induced permeability makes it a region of interest for future investigations and the targeting of treatment paradigms.

Biological Sources of Variance

We began to address potential biological sources by allowing the mice ad libitum access to both food and water until euthanasia. Chief amongst biological sources of variance was the anatomical region from which the segment was made. Our data show that tissue from the gastric regions of (duodenum and jejunum) is more permeable and susceptible to heat-induced barrier disruption that the distal ileum. Further investigation is required to differentially analyze regions of the small bowel to elucidate the mechanisms controlling the susceptibility of the gastric regions.

Previously, our lab analyzed the effect of the anatomical regions from which segments were created on permeability values. We did not observe a significant difference in permeability between gastric and ileal regions of the small bowel. This was supported by Lambert et al's findings that permeability did not vary, at baseline or with heat, as a function of intestinal location (Lambert, 2002). In my analyses, however, I found a significant and proportional relationship between permeability and anatomical region. Segments in the gastric region of the small intestine appear more susceptible to permeability increases, both at baseline and with heat. This is supported by Yu et al's (Yu, 2010) *in vivo* findings of a similar relationship of increased damage in gastric regions after long term heat exposure. Morphological alterations in epithelial ultrastructure following three (3) days of exposure were most severe in the jejunum. Microvillus height was significantly shorter in heated jejunum compared to control; no difference was found in ileal tissue. The jejunal epithelium also had a higher number of damaged mitochondria and showed more organelle debri within lysosomes (Yu, 2010). We saw regional differences within the two (2) hours of our protocol. It is possible that this effect may have been previously indecipherable in our covered-cuvette protocol because of the extreme variance of the other factors.

Origins of Biological Variation

The susceptibility of this region to damage may be explained by fact that the protective mucus layer of the digestive tract is already thinnest in the small intestine (Allen, 2005). This, when combined with larger crypt-to-villus ratios in the proximal small intestine, likely to increase absorptive capacity, provides a larger surface area of exposed epithelial cells for potential damage. This may explain why the regional differences in permeability appear exacerbated with the heat stress. Also of interest is

the regional expression of tight junction proteins; the number of junctional strands correlates with junctional tightness (Steed, 2010) and deficient expression may increase susceptibility to permeability. Regional differences in drug or sodium transporters along the intestine (Englund, 2006) provide possible explanations for the varying susceptibility. These regulators of transcellular transport regulate the flux of water into the lumen have the potential to drive paracellular solute diffusion across tight junctions (Turner, 2009). Regional susceptibility to damage, *in vivo*, may be attributed to fully active digestive enzymes emptied directly into the duodenum from the stomach and pancreas (Allen, 2005). Potential proteolytic degradation of epithelial cells compounds the potential effects of increased heat on barrier function (Schmid-Schonbein, 2008).

Critique of Isolated Segment Model

The development of this *in vitro* segmental model of intestinal susceptibility to hyperthermia is invaluable to our laboratory and to our understanding of epithelial function. It allows the efficient testing of potential treatments for hyperthermia along the length of the intestine. However, in the body, increases in core temperature are associated with intestinal ischemia, a facet of hyperthermia currently missing from our model. Furthermore, we question the sensitivity of our system, as we have previously been unable to successfully increase tissue permeability with compounds known to initiate tight junction opening. These compounds have yet to be tested with our improved methodology. The method of gut sac preparation is also likely to disrupt the integrity of the adherent mucus layers, normally responsible for mucosal protection.

In our attempt to physiologically explore the mechanisms of intestinal dysfunction in hyperthermia, we have addressed these shortfalls in the development of multiple functioning models of intestinal analysis. Our laboratory has since developed

functioning anesthetized and unanesthetized whole animal models of heat stress. Throughout either of these courses of research it will be important to examine the regional status of barrier properties to more physiologically explore the regional intestinal permeability identified in our *in vitro* model.

Conclusion

The gastric regions of the small intestine are more susceptible to heat damage. This results in an increased danger of unregulated solute movement and runaway inflammatory and immune responses. Moreover, the variation noted along intestinal length serves as a caveat for investigators seeking the universal application of findings from manipulations of only a single region.

The regional differences in similarly treated tissues in our model highlight the importance of considering the entire intestinal tract in investigations. It provides insight into the relative importance of Schmid-Schonbein‘s auto-digestion theory, which traces the multi-organ failure that is symptomatic to shock conditions, such as occurs in heat stress, to fully active digestive enzymes being emptied directly into the duodenum (Schmid-Schonbein, 2008). Pancreatic enzymes have the power to degrade most biological molecules and under normal conditions, the epithelium is protected, in part, by mucus-bicarbonate secretion. Under ischemic conditions however, the intestinal wall becomes permeable to these enzymes, exacerbating the mucosal injury in heat stress and stimulating the over-activation of the production of inflammatory mediators.

The identification and control of mechanisms regulating the preservation of tight junctions and epithelial cell viability are needed to direct efficacious environmental, nutritional, or pharmaceutical interventions. Our data suggest that interventions for heat-related illnesses may be most effective when targeted to gastric regions of the

small intestine. Other important areas for future research include the bacterial-epithelial interactions through toll-like and other receptors, the mechanisms for the sensing of ischemia and those regulating mitochondrial membrane potential, and the real-time assessment of cellular and subcellular structures related to mucosal defense.

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BIOGRAPHICAL SKETCH

Neil has been involved with Dr. Thomas Clanton's laboratory for just over a year. At the commencement of his scientific career there, his work centered mainly on the development of a genetically-encoded calcium indicator that will allow the detection of calcium transients and rapid action potentials in excitable cells.

Since then, he has worked on the development and refinement of an *in vitro* mouse model of investigating the mechanisms of heat-induced intestinal barrier dysfunction. During this time, he came across the novel finding that the distal region of the mouse small intestine is less susceptible to hyperthermia-induced permeability increase. One possible application of this information is the directing of heat-related illness treatments to the gastric regions of the small intestine. To further explore this, he will begin testing tissue samples from unrestrained, unanesthetized mice that have been allowed to experience increased core temperatures. The development of a viable whole-body heat stress model will allow his lab to investigate regional intestinal sensitivity in an intact animal system.

After the completion of his doctoral program in Applied Physiology and Kinesiology, Neil plans to achieve his childhood goal of procuring a medical school education. He is certain that the skills and knowledge garnered throughout his graduate experience will solidify my competence as a scientist and his role in increasing our understanding of the workings of the human body.