Three Gastrointestinal Assays

A Senior Honors Thesis

Submitted in Partial Fulfillment of the Requirements
for Graduation in the Honors College

By

Bailey Majtyka
Biology and Psychology Major

The College at Brockport

May 2, 2018

Thesis Director: Dr. Adam Rich, Associate Professor, Biology

Abstract

Gastrointestinal (GI) functionality relies on the spontaneous, rhythmic and coordinated propagation of muscular contractions in the GI tract, or GI motility. Without these coordinated motor patterns, digestion falters, and results in problems with digestion. Disrupted or un-coordinated motor patterns are associated with altered GI transit times. GI transit is the amount of time necessary for intestinal contents to move through the GI tract. GI transit is measured in patients complaining about abdominal discomfort to determine if discomfort results from a true dysmotility or from idiopathic symptoms. GI transit assays help to determine appropriate treatments but idiopathic symptoms, or pain from an unknown cause, is very common. The zebrafish is an attractive model system for human GI motility because the entire GI tract can be observed in intact zebrafish larva. In current methods, larvae are fed food with a marker substance and movement through the intestine is viewed using a microscope and recorded using a digital camera. However, GI transit time is highly variable. It is possible that this variability is completely normal and results from variable GI physiology. Alternatively, it is possible that the variability is due to the assay. Three distinct GI transit assays have been published. The overall objective for this work is to determine the reliability for each assay and to better understand which assay is most appropriate for future work. The assays will be described and compared, and results comparing the assays will be presented.

Introduction

The gastrointestinal tract is a series of organs that allows for the digestion of food and elimination as waste. The simplest description of the organs are the mouth, esophagus, small intestine, large intestine, colon, rectum, and anus. The entire human GI tract spans over thirty feet long from mouth to anus with a total mucosal surface area measuring to approximately 32 m^2, depending on the size of the individual (Helander and Fändriks, 2014). If the surface area could be laid flat it would require enough room to park 5 cars! The whole of the tract is innervated by various neuroendocrine components that assist in regulating motility and digestion, as well as a pacemaker system to initiate contractions. Digestion requires secretion and absorption, and motility is matched with absorption to optimize nutrient absorption (Liao et al. 2009). It is important to understand that without motility bacteria in the GI tract multiply rapidly. These bacteria secrete toxins which influence the mucosal and epithelial lining of the GI tract and can cause the gut to 'leak' toxins into the blood. This condition is called sepsis and is life threatening (Carl et al. 2014). Many dysmotility disorders, however, merely reduce the quality of life for patients and are associated with severe discomfort (Böhn et al., 2013). The GI tract contains an impressively large system of organs, and an equally impressive number of possible problems that can occur.

According to data collected by the National Institute of Health, GI problems cost America approximately 142 billion dollars each year based on a study conducted in 2004 (National Institute of Diabetes and Digestive and Kidney Diseases, 2018). Additionally, nearly 25% of Americans had some form of GI problem in 2015 (National Institute of Diabetes and Digestive and Kidney Diseases, 2018). Of keen interest is the prevalence of GI distress of patients following surgery and patients in the ICU. Among critical care patients, the prevalence

rate of GI complications can range from 50 to 80% (Adike and Eamonn, 2014). This is of concern due to the possibility of lengthening the time spent in the hospital, the increased risk of infection, and even increased mortality rates (Adike and Eamonn, 2014). Additionally, patients undergoing chemotherapy often have GI distress (Carbone, et al. 2016). Problems associated with chemotherapy are often due to problems with GI transit (Helander and Fändriks, 2014).

The study of GI motility is important due to the various health effects associated with irregularities in digestive systems, and especially because of the prevalence of motility disorders (National Institute of Diabetes and Digestive and Kidney Diseases, 2018). Motility disorders range in severity and in symptoms. Some motility disorders, but not all, influence the natural progression of intestinal contents and therefore cause negative changes in digestion. Common diseases associated with irregularities in GI motility include Crohn's Disease, gastroenteritis and irritable bowel syndrome (IBS) (Yarandi and Srinivasan, 2014, Hahnemann, 2015). These problems in GI motility may affect or be affected by the three key intrinsic regulatory mechanisms in the gut: interstitial cells of Cajal, smooth muscle autoregulation, and innervation by enteric neurons (Brady et al. 2017). The irregularities that cause these diseases can lead to GI failure, or the inability of the GI tract to contract and propel contents effectively (Kubicki and Warrillow, 2018). GI failure is associated with negative care outcomes, increasing a patient's mortality and morbidity rates when comorbid with other illnesses (Reintam et. al, 2006).

Components of GI Motility

Interstitial cells of Cajal (ICC) generate slow waves that are responsible for regulating chronotropicity and ionotropicity (Lyford and Farrugia, 2003). Chronotropicity refers to the timing of contractions and ionotropicity refers to contraction strength. Put more simply, ICC help control how quickly contents are propelled and how strong the contractions are along the GI tract

by sending signals to surrounding muscle cells necessary for contracting the intestinal walls. The ICC method of action revolves around the use of a calcium ion channel that will open after exposure to a mechanical stimulus (Lyford and Farrugia, 2003). A mechanical stimulus may be the digesting food that is moving along the tract. When a mechanoreceptor is stimulated, the ion channel will open and will allow calcium entry into the ICC. Calcium entry in turn stimulates a series of events, ultimately resulting in membrane depolarization. This electrical event within ICC is transmitted to smooth muscle cells by gap junctions, direct connections to smooth muscle cells that allow electrical current to flow (Al-Shboul, 2013). In smooth muscle electrical depolarization also stimulates calcium channels to open, and this leads to muscular contraction. ICC coordinates the contractions of multiple smooth muscle cells in the region surrounding them. The electrical signal can spread 'forward' more quickly though ICC stimulating its adjacent region. This process repeats itself as the signal travels, resulting in a propagating contraction ring long the GI tract in an oral to anal direction (Al-Shboul, 2013). It is obvious that there are many components in this mechanism that allows for spontaneous and rhythmic contractions to develop and to propagate, and ICC are one of these regulators. The coordination, speed, and force of intestinal smooth muscle contraction are easily visualized as the contraction wave propagates through the tract, in large part due to ICC (Huizinga et al., 1997).

Enteric neurons can be excitatory and increase contraction force, or inhibitory, and decrease contraction force. Enteric neurons can transmit signals directly to ICC, and also directly to smooth muscle. Enteric neurons are part of the enteric nervous system (ENS) which is separate from the central nervous system (Rao and Gershon, 2016). The ENS is largely independent and can autoregulate GI motility without input from the central nervous system, but may still be influenced by the autonomic branch of the central nervous system (Rao and

Gershon, 2016). The role of the ENS is crucial for GI motility. If enteric neurons are missing in just one subsection of the GI tract, it is possible the GI transit can stall, and the tissue may no longer propel contents along the denervated portion of intestine. This can lead to further health complications, such as Hirschsprung's disease (Chhabra and Kenny, 2016). Intestinal contents do not pass the affected region in Hirschsprung's patients resulting in a buildup of intestinal contents upstream and adjacent to the affected region. This is caused by the aganglionic region of the GI tract, the region that lacks ENS, being contracted and creating the obstruction. Therefore, the inhibitory innervation associated with the ENS must be essential to its function.

The enteric nervous system has been considered to be a second brain in the gut by some sources (Nezami and Srinivasan, 2010). The ENS is divided into two subsets, the myenteric and submucosal plexus, each with differing functions and are located in separate layers along intestinal walls (Nezami and Srinivasan, 2010). The enteric neurons in the myenteric plexus innervates smooth muscle and ICC, assisting in regulation and coordination of muscular contractions (Mazzuoli and Schemann, 2012). Additionally, the submucosal layer assists the mucosa in secretions and innervates the myenteric plexus through a more neuroendocrine role (Nezami and Srinivasan, 2010). Enteric neurons also serve as an important feedback pathway for the GI tract. The sensory neural signals provide the central nervous system with information regarding GI motility and the central nervous system can provide the ENS with instructions (Altaf and Soof, 2008).

Smooth muscle cells have the ability to autoregulate, meaning that they can function independently (Scirocco et al. 2016). Smooth muscle cells respond to changes in intracellular calcium concentration that result from mechanically induced calcium influxes (Sanders and Koh, 2007). For example, stretch activated channels are activated by the plasma membrane being

stimulated by the pressure of luminal contents pressing against the channel's mechanoreceptors. This causes an increase in intracellular calcium concentration which triggers smooth muscle contraction, propelling contents further along the GI tract (Sanders and Koh, 2007). Smooth muscles cells also respond to hormones and neuroendocrine factors that can increase or decrease contractile force (Sanders and Koh, 2007). Cytokines appear to be of key interest in understanding pathology associated with smooth muscle cells (Scirocco et al. 2016). Their inflammatory properties have varying effects on smooth muscle tone, causing increased or decreased contractility (Ohama et al., 2007). This effect varies based on the region of the gut that is associated with the inflammation and is therefore exceedingly complex to study. This process is one explanation as to why diseases like inflammatory bowel disease can cause changes in transit time (Ohama et al., 2007). Additionally, psychosocial stress has been linked with an increased production of cytokines in humans (Maes et al., 1998). However, there has not yet been a causal link associated with the psychosocial behavior inherently causing the gastrointestinal problems (Shah et al., 2014).

Gastric emptying and transit time of intestinal contents are important indicators of gastrointestinal motility because movement of the luminal contents reflects the integrated activity of ICC, ENS, and smooth muscle cells. Diseases affecting GI motility that are associated with abnormal GI transit times include IBS, inflammatory bowel disease, ileus, and gastroparesis (Haase et al. 2016, Törnblom et al., 2011, Tsukamoto et al., 1999). Therefore, an effective way to study GI motility is to measure transit time (Field et al., 2009, Cassar et al., 2015, Zhou et al., 2014). Transit time is defined as the time required for luminal contents to progress through a GI tract and to be expelled by an organism. Utilizing this concept, it will be possible to determine

the effect of missing factors associated with GI motility such as: interstitial cells of Cajal, smooth muscle, and enteric neurons.

Zebrafish Model

The zebrafish model is an invaluable asset to researchers. A key trait associated with the organism is its natural transparency during its embryonic stage which allows direct imaging of GI motility in intact larva (Rich, 2009). This transparency can be lengthened through gene manipulation or soaking zebrafish larvae in 1-phenyl-2-thiourea (PTU) (Rich, 2009, Field et al. 2009). Transparency is an attractive quality of the model because it allows researchers to study organ function without dissection, or invasive sample collections (Rich, 2009). Additionally, zebrafish development occurs rapidly, becoming sexually mature as early as three months after birth (Fontana et al. 2018). The zebrafish GI tract has also been confirmed to have developed their fully functional regulatory mechanisms, ICC and ENS, by 7 days post-fertilization (dpf) (Rich, 2009). This allows researchers to fully visualize the GI tract's propagation of intestinal contents through the animal very early on in its development. However, while the GI tract in the zebrafish model is simpler than a human's, it still comprises the three main regulatory mechanisms, ICC, ENS, and smooth muscle (Rich, 2009). Furthermore, the zebrafish model has a fully mapped genome that is 70% similar to human (Fontana et al. 2018). This has been exploited by a multitude of researchers, as the development of transgenic animals progresses today (Udvadia and Linney, 2003). Zebrafish can be easily manipulated to study components thought to be critical to healthy physiology and treatment measures can be assessed (Brady et al. 2017). Of key interest for this thesis are GI transit assays and how their implementation could be optimized for future research studies.

Gastrointestinal Transit Assays

Three published zebrafish transit assays will be analyzed in the current study. Assays developed by Field et al., Cassar et al., and Zhou et al. have all been reported as reliable, effective methods of analyzing gastrointestinal transit (Field et al., 2009, Cassar et al., 2015, Zhou et al., 2014). Each transit assay involves the use of an indigestible fluorescent tracer that will fill the zebrafish's intestinal bulb. The tracers are ideal in that there is a high signal to noise ratio and the tracers are not absorbed. A high signal to noise ratio means that it is easy to measure very small amounts of the compound. It is essential that the tracer is not absorbed along the GI tract, otherwise it becomes more difficult to see and track the luminal contents within the zebrafish larva. The tracer may be ingested by the organism through a tracer-dry food mix or by adding a fluorescent solution to the fish water which is swallowed. The assays report how the tracer moves through the GI tract of a 7dpf larva. Digital images may then be captured using fluorescent microscopy at the time of loading, at the 4-hour mark, and 24 hours following the first reading.

We used drug controls to compare the three different assays. These drug controls consisted of prokinetics, drugs that will increase GI motility and will decrease transit time. These drugs should decrease the amount of time it takes for the fluorescent tracer to move through the zones in the Field Assay, decrease the total fluorescence in the Nile Red assay more quickly, and cause an increase in the rate of fluorescent buildup at the bottom of the well in the Cassar assay when compared to wild type and vehicle agent controls. Drugs that slow GI motility will increase transit time. This should lead to a longer amount of time for the tracer to move through the zones in the Field Assay, larger fluorescence recordings in the Nile Red Assay, and decreased fluorescence buildup at the bottom of the wells in the Cassar Assay. This project focuses on

analyzing and assessing the GI transit assays through the use of three drug controls dissolved in a dimethyl sulfoxide solvent (DMSO). These assays are expected to provide similar data, provided they are reliable tools. The results from this study will provide pertinent information that can be implemented into future experiments.

Materials and Methods

Aquaculture

Wild-type zebrafish from the Zebrafish International Resource Center were maintained in the lab in accordance with the Institutional Animal Care and Use Committee guidelines. Zebrafish were fed dry food twice each day (Aquaneering). The zebrafish were bred in the morning and embryos were maintained in embryo medium (E2) in petri dishes (~25 embryos/dish) in an incubator set to 28 degrees Celsius with a 14-h light/10-h dark cycle (Brady et al. 2017). Embryos were transferred to clean dishes daily with fresh media. Beginning at 5 dpf, larvae were fed hatch fry encapsulation, grade 0, by gently blowing powdered food using a Pasteur pipette onto the media to avoid overfeeding (Argent, Redmond, WA).

Assay One: *Field Assay*

The assay developed by Field et al. (hereby referred to as the Field Assay) revolves around a fluorescent tracer's most proximal location in the GI tract (Field et al. 2009). The tracer is mixed with dry food and presented to 7 dpf zebrafish one hour before initial measurements. This gives the zebrafish ample time to feed. This proximal location is the end of the tracer in the most oral direction. Over the course of the 24-hour transit assay, the tracer will progress through the GI tract in a healthy animal. This assay divides the GI tract into four zones and records the location of the most oral segment of tracer at each time point. These measurements will be recorded at 0, 4, and 24-hour marks. At each time point zebrafish must be anesthetized in a 1X

tricaine solution and imaged using a fluorescence microscope. The data recorded will indicate how quickly the tracer is being propelled through the GI tract. Tracer movement over time can be used to assess GI motility. This methodology should be sensitive to changes in GI motility, whether it be due to drug or due to anatomical changes in the zebrafish sample (Field et al. 2009).

Assay 2: *Nile Red Assay*

The concept behind the Nile Red assay developed by Zhou et al. is centered on the idea of a fluorescent tracer solution filling the entirety of a zebrafish sample's GI tract. It differs from the Field Assay because the tracer is dissolved in E2 and dye loading does not require feeding. After soaking in a Nile Red fluorescent tracer solution over the course of 24 hours, a 6dpf zebrafish will have ingested enough fluorescent tracer to completely fill the zebrafish GI tract. Measurements are then performed at 7dpf. Prior to data collection, zebrafish are anesthetized in 1X tricaine mixed in E2 before being laterally positioned for optimal viewing of the GI tract. The anesthetized zebrafish is immobilized by covering it with a 3% Agarose solution warmed to 40 degrees Celsius. The zebrafish may then have their intestinal fluorescence recorded at each time point with fluorescent microscopy. Zebrafish larvae were examined using an Olympus BX 51 microscope and images were captured using a QImaging Retiga camera with a cooled CCD using Image Pro Plus software (Media Cybernetics, Rockville, MD). Following image capture, regions of interest were drawn around the GI tracts of each zebrafish and produced a total number of pixels and fluorescence intensity. This value, the total fluorescence, decreases at each time point and is a measure of GI transit. When exposed to varying drug treatments, there will be an observable effect on the gastrointestinal fluorescence degradation (Zhou et al. 2014).

Cassar Assay

The Cassar Assay is similar to the Field assay in that the zebrafish sample is fed the fluorescent tracer in a food mixture. After feeding three 7dpf zebrafish per well are deposited. The zebrafish fecal matter collects at the bottom of the wells. Accumulation of fluorescent tracer is measured using a fluorimeter. Measurements may be made at the 0-hour, 4-hour, and 24-hour marks. These experiments can be automated and performed in a 96 well plate, ideal for medium to high throughput experiments. The fluorescence data that is collected can be used to make conclusions regarding drug treatments, pathophysiology, or other induced treatments (Cassar et al. 2015). Experiments with this Assay were not conducted.

Fluorescent Tracer Food and Fluorescent Dye Solutions

In the Field assay, larvae were allowed to feed for 1 hour with finely powdered dry food (Argent, 30 μm particles) labeled with rhodamine B-marked melamine beads (Sigma, 1 μm, emission maximum ~584 nm). Labeled food was prepared the night before experiments and stored in a dark, dry drawer before feeding.

Fluorescent dye Nile Red was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as a dissolved tracer to visualize the zebrafish GI transit. Nile red dye was mixed with E2 at a final concentration of 10 μg/L. A 1 mg/L stock solution was stored in a dark refrigerator and was diluted prior to use.

Additional Treatment Procedures

Between time points during both the Nile Red and Field Assay procedures, larvae are removed from the tricaine wells. After exposure to the tricaine, each larva is returned to warmed embryo medium without Tricaine. Following rescue, the zebrafish are placed back into their individual wells or petri dishes in preparation for the next time point. Following the final data

collection of each procedure the zebrafish samples were sacrificed using a Tricaine overdose in ice cold embryo medium.

Drug Controls

Table 1. Drug and Solvent Controls Used

Controls Used					
Positive Controls	Concentrations	Negative Control	Concentrations	Solvent Control	Concentration
Domperidone	30mg/L	Atropine	4.2 µM	DMSO	0.1%
	100mg/L				
Mosapride	10 mg/L				

Domperidone is a prokinetic. It increases GI motility by enhancing peristalsis along the GI tract through the blockage of these dopamine receptors (Silvers et al. 1998). Domperidone has a strong affinity for D2 and D3 receptors (Silvers et al. 1998). Domperidone (Sigma-Aldrich, St. Louis, MO, USA) was used at two different concentrations in the study, 30 mg/L and 100 mg/L. It was dissolved in DMSO.

Mosapride is also a prokinetic and is a selective serotonin receptor agonist. It stimulates 5-HT4 resulting in acetylcholine release (Lim et al, 2013). Mosapride (St. Louis, MO, USA) was used at a 10 mg/L concentration and was dissolved with a DMSO solvent. A stock solution was maintained at a 100 mg/L.

Atropine decreases GI motility and slows GI transit by reducing the total number of muscular contractions. It is an anti-parasympathetic drug that operates by inhibiting muscarinic receptors and is suspected to have an effect on M1 and/or M3 muscarinic receptors in smooth muscle cells (Collares et al. 2017). Atropine (St. Louis, MO, USA) was utilized at a 4.2µM concentration and dissolved using a DMSO solvent prior to mixing within an E2 solution. Atropine stock solution was maintained at 42 mM in DMSO.

Control experiments were performed to verify the motility effects do not result from DMSO alone. 0.1% DMSO solvent was utilized as a vehicular control. the highest levels of DMSO in our solutions was 0.01%. This ensured that observable effects are due to the drug and not from DMSO alone. Initial experiments compared DMSO containing E2 with E2.

Statistical Analysis

The Field Assay required a non-parametric analysis because tracer position was recorded within zones. Averaging of zones was unacceptable because a zone value must be discrete, meaning it must be a value of 1, 2, 3 or 4. A non-discrete zone value resulting from averaged data would be meaningless. We selected the nonparametric Kolmogorov-Smirnov test. The wild type controls were held constant in each individual study. These samples served as a basis for analyzing the experimental groups associated with each drug treatment. In the Nile Red assay, a simpler two tailed t-test was used because data was not discrete.

Results

The Field assay measures dye movement during 24 hours after feeding. Dye was not present in some larvae during the first time point and these larvae were excluded from analysis. The position of dye within the GI tract as measured at 4 hours, and at 24 hours after the end of feeding. Figure 1 shows the position of the dye in vehicle- treated larvae, and the effects of two control compounds. Atropine inhibits cholinergic neurotransmission and is a well characterized drug that inhibits GI motility. Domperidone inhibits dopamine receptors and it increases GI motility. Domperidone is used clinically to treat nausea and vomiting. GI transit is shown in the figures as a progression of the dye from Zone 1, which is classified as dye within the intestinal bulb, to Zone 4, which is classified as dye near to the anus. Zone 5 is chosen when no dye is visible, indicating that all of the dye has been eliminated from the intestine. For control larvae

(grey bars) the dye is distributed in nearly all of the zones at 4 hours, and at 24 hours the majority of larvae have eliminated the dye (zone 5). DMSO did not affect GI transit (first column), whereas Atropine delayed GI transit Middle Column). Domperidone had no effect (third Column).

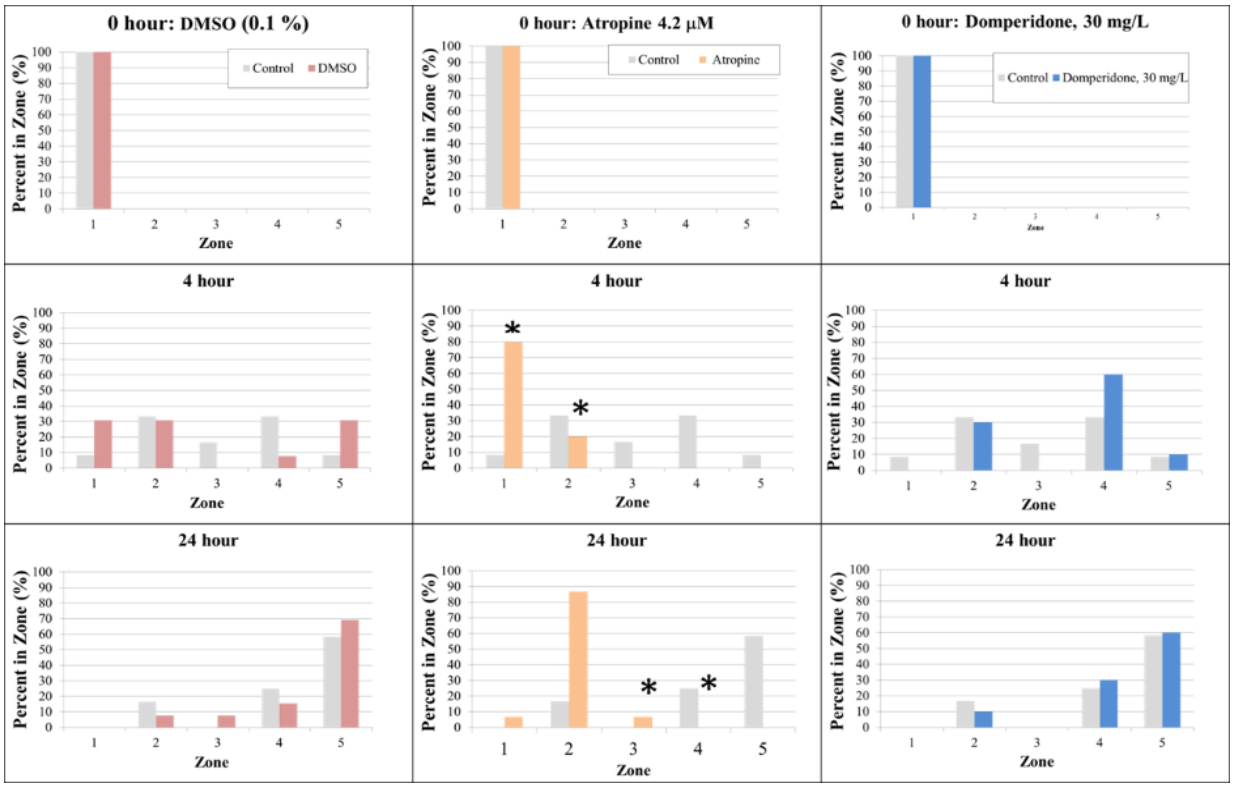

Figure 1. Effects of Atropine and Domperidone on GI transit in 7 dpf larvae. Atropine slowed transit, shown in the 4 hour time point (tan bars) when compared to DMSO treated control larvae. Domperidone had no effect.

A second control experiment was performed using a higher concentration of Domperidone (100 mg/L, the prokinetic drug Mosapride, and the Sparse mutant zebrafish. Mosapride is a serotonin receptor agonist and is used clinically to treat dyspepsia. Sparse mutants lack Kita, and have a decreased frequency and disorganized coordination for GI motility

(Rich et al, 2007). Both high dose Domperidone and Mosapride were without effect on GO transit. The Sparse mutant had a statistically significant reduction in GI transit.

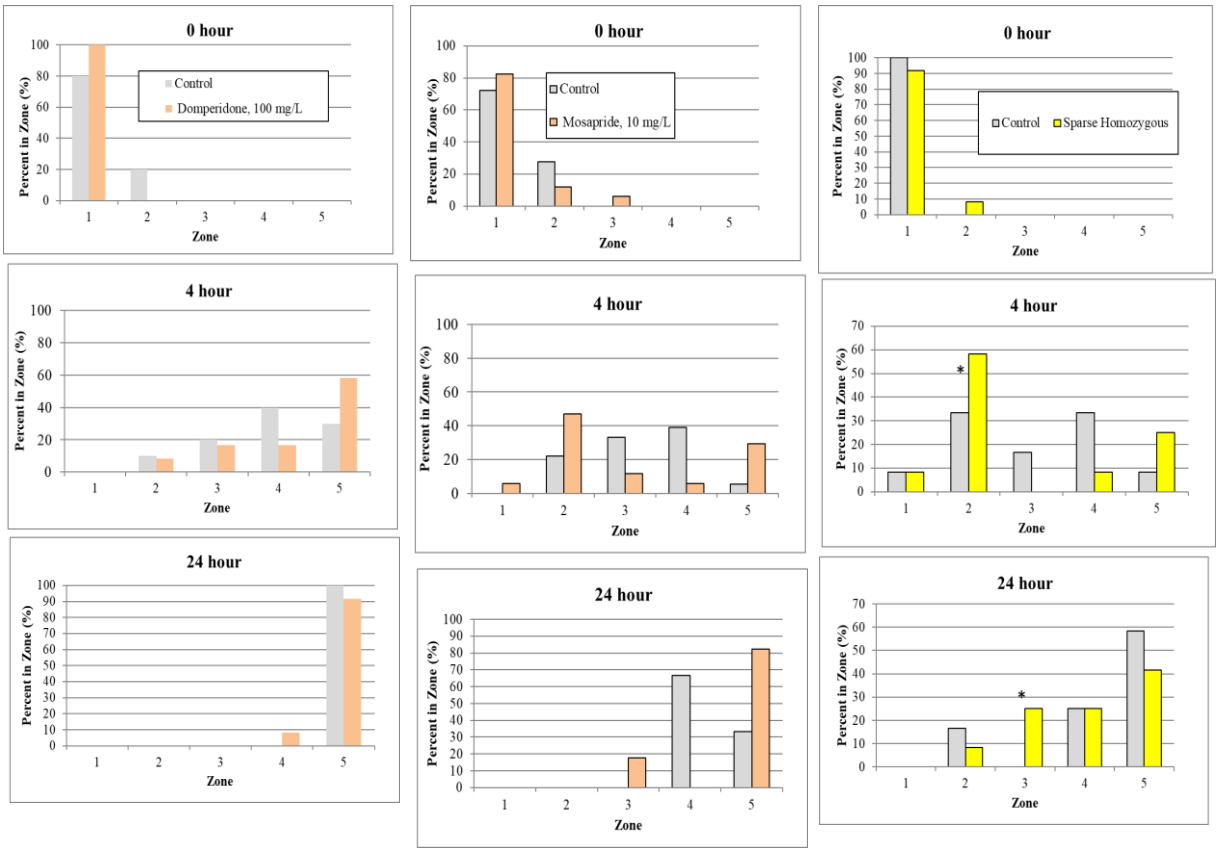

Figure 2. Effects of high-dose Domperidone, Mosapride, and the Sparse mutant on GI transit. Higher doses of Domperidone, the prokinetic had no effect on GI transit. Mosapride, another prokinetic agent, had no effect on GI transit. The Sparse mutant showed delayed transit compared to controls.

The Nile Red assay fills the GI tract with a marker dye that is dissolved in the water the fish swim in. At specific time points an image, a region of interest placed over the intestine, and average fluorescence intensity is calculated. The average fluorescence intensity decreased with Mosapride compared to vehicle control (left panel). The average fluorescence intensity also decreased at 4 hours with Domperidone compared to control (right panel). These results are

consistent with an increase in GI transit, as expected for prokinetic drugs. Interestingly DMSO appears to slow GI transit in one set of experiments (right panel).

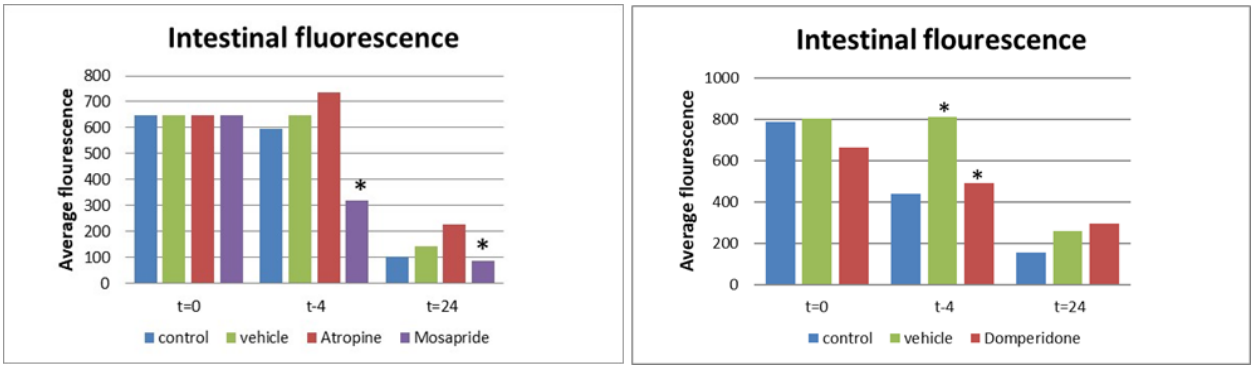

Figure 3. GI motility measured using the Nile Red assay. Fluorescence was maximal at the first time point taken immediately after larvae rinsing to remove Nile red. Atropine and mosapride

Discussion

Domperidone decreased GI transit time, consistent with increased GI motility, at a dose of 100 mg/L. This was measured using the Field assay and the Nile red assay. Lower doses were ineffective. The Nile Red assay was the best assay in that it reported changes in GI transit time amongst all drugs. In other studies, both assays have been shown to be capable of reliably assessing gastrointestinal transit time in 7 dpf larvae (Field et. al, 2009, Zhou et al. 2014).

The Field assay requires the use of a large sample size to see a significant effect. In small samples, there will likely be no observable effect. If there is an observable effect with a small sample, it is possible that the data collected may be skewed by natural GI transit variability. A weakness in this methodology is after the return to the treatment dish, individuals within the sample are unable to be tracked during the course of the study. This can be easily addressed by placing individual zebrafish in separate wells to be tracked over the course of the experiment. Additionally, there is no guarantee that the larva will all eat the fluorescent tracer and completely

fill their intestinal bulb. This reduces the total number of zebrafish eligible for study. Some compounds may reduce feeding and it may be predicted that drugs interacting with GI motility may influence feeding behaviors. Additionally, it is possible for instrumental error associated with the observer conducting the procedure. Despite having established zones of measurement for the fluorescent tracer, it is possible that different observers will have different interpretations on where the most proximal position of the fluorescent tracer actually is. However, a strength of this study is the relative ease in measuring each zebrafish and the ability to measure many zebrafish, provided they meet the selection criteria.

The Nile Red Assay is an attractive method to measure a small number of zebrafish samples. However, as GI variability is common, it is possible for an outlier in a small sample size to skew the data. Additionally, this method was labor intensive, requiring more time than the Field assay to prepare for experiments, to conduct the experiments, and analyze the data following the experiments. Furthermore, the region of interest in the imaging can be a challenge to identify. If future experiments utilizing the Nile Red assay is utilized, it is highly recommended that PKU be utilized on the zebrafish samples to reduce pigmentation. The pigmentation in the 7dpf fish interferes with the total fluorescence that was being picked up in the region of interest drawn around the GI tract.

I was unable to perform the Cassar Assay. Data collected from this procedure will need to be analyzed and compared to those of the Holly Field and Nile Red Assay. There also needs to be additional drug controls. While the domperidone, mosapride, and atropine controls allowed for trends to be seen between the two assays, additional drug controls would provide additional information regarding the assays' reliability.

Ideally, future experiments will include continuous recording of the gastrointestinal tract during the entire digestive period in un-anesthetized larva. This will be challenging because larva will move and therefore data collection and/or analysis will be complicated. Continuous recording will eliminate problems associated with discrete time intervals, and with repeated anesthesia which is likely to have undesirable effects on larva health and on GI function. A continuous recording assay is currently being developed by Adam Rich and Jon Sleeper in an effort to address this limitation.

Works Referenced

1. Adike, Abimbola and Eamonn M M Quigley. "Gastrointestinal Motility Problems in Critical Care: A Clinical Perspective." *Journal of Digestive Diseases*, vol. 15, no. 7, July 2014, pp. 335-344
2. Altaf, Muhammad A and Manu R Sood. "The Nervous System and Gastrointestinal Function." *Developmental Disabilities Research Reviews*, vol. 14, no. 2, 2008, pp. 87-95
3. Al-Shboul, Othman A. “The Importance of Interstitial Cells of Cajal in the Gastrointestinal Tract.” *Saudi Journal of Gastroenterology : Official Journal of the Saudi Gastroenterology Association* 19.1 (2013): 3–15. *PMC*. Web.
4. Böhn, L., Störsrud, S., Törnblom, H., Bengtsson, U. and Simrén, M. (2013). Self-Reported Food-Related Gastrointestinal Symptoms in IBS Are Common and Associated With More Severe Symptoms and Reduced Quality of Life. *The American Journal of Gastroenterology*, 108(5), pp.634-641.
5. Brady, Clayton, et al. "Intestinal Transit Time and Cortisol-Mediated Stress in Zebrafish." *Zebrafish*, vol. 14, no. 5, Oct. 2017, pp. 404-410. EBSCO*host*, doi:10.1089/zeb.2017.1440
6. Carbone, S E, et al. "Electrophysiological and Morphological Changes in Colonic Myenteric Neurons from Chemotherapy-Treated Patients: A Pilot Study." *Neurogastroenterology and Motility: The Official Journal of the European Gastrointestinal Motility Society*, vol. 28, no. 7, July 2016, pp. 975-984
7. Carl, Mike A. et al. “Sepsis From the Gut: The Enteric Habitat of Bacteria That Cause Late-Onset Neonatal Bloodstream Infections.” *Clinical Infectious Diseases: An Official*

*Publication of the Infectious Diseases Society of America*58.9 (2014): 1211–1218. *PMC*. Web.

8. Cassar S, Huang X, Cole T. A high-throughput method for predicting drug effects on gut transit time using larval zebrafish. J Pharmacol Toxicol Methods 2015;76:72–75.
9. Chhabra, S. and Kenny, S. (2016). Hirschsprung's disease. *Surgery (Oxford)*, 34(12), pp.628-632.
10. Collares, E.F., A.M. Vinagre, and C.B. Collares-Buzato. "Dipyrone in Association with Atropine Inhibits the Effect on Gastric Emptying Induced by Hypoglycemia in Rats." *Brazilian Journal of Medical and Biological Research*50.10 (2017): e5948. *PMC*. Web
11. Field, H A, et al. "Analysis of Gastrointestinal Physiology Using a Novel Intestinal Transit Assay in Zebrafish." *Neurogastroenterology and Motility: The Official Journal of the European Gastrointestinal Motility Society*, vol. 21, no. 3, Mar. 2009, pp. 304-312.
12. Fontana, B., Mezzomo, N., Kalueff, A. and Rosemberg, D. (2018). The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review. *Experimental Neurology*, 299, pp.157-171.
13. Haase, A., Gregersen, T., Christensen, L., Agnholt, J., Dahlerup, J., Schlageter, V. and Krogh, K. (2016). Regional gastrointestinal transit times in severe ulcerative colitis. *Neurogastroenterology & Motility*, 28(2), pp.217-224.
14. Hahnemann, M L, et al. "Quantitative Assessment of Small Bowel Motility in Patients with Crohn's Disease Using Dynamic MRI." *Neurogastroenterology and Motility: The Official Journal of the European GI Motility Society*, vol. 27, no. 6, June 2015, pp. 841-848

15. Helander, Herbert F and Lars Fändriks. "Surface Area of the Digestive Tract - Revisited." *Scandinavian Journal of Gastroenterology*, vol. 49, no. 6, June 2014, pp. 681-689. EBSCO*host*, doi:10.3109/00365521.2014.898326.
16. Huizinga, J., Thuneberg, L., Vanderwinden, J. and Rumessen, J. (1997). Interstitial cells of Cajal as targets for pharmacological intervention in gastrointestinal motor disorders. *Trends in Pharmacological Sciences*, 18(10), pp.393-403.
17. Kubicki, M. and Warrillow, S. (2018). Gastrointestinal problems in intensive care. *Anaesthesia & Intensive Care Medicine*, 19(3), pp.93-97.
18. Liao, Dong-Hua, Jing-Bo Zhao, and Hans Gregersen. "Gastrointestinal Tract Modelling in Health and Disease." *World Journal of Gastroenterology : WJG*15.2 (2009): 169–176. *PMC*. Web.
19. Lim, Hyun Chul et al. "Effects of the Addition of Mosapride to Gastroesophageal Reflux Disease Patients on Proton Pump Inhibitor: A Prospective Randomized, Double-Blind Study." *Journal of Neurogastroenterology and Motility* 19.4 (2013): 495–502. *PMC*.
20. Lyford, G. and Farrugia, G. (2003). Ion channels in gastrointestinal smooth muscle and interstitial cells of Cajal. *Current Opinion in Pharmacology*, 3(6), pp.583-587.
21. Maes, M., Song, C., Lin, A., De Jongh, R., Van Gastel, A., Kenis, G., Bosmans, E., De Meester, I., Benoy, I., Neels, H., Demedts, P., Janca, A., Scharpé, S. and Smith, R. (1998). The effects of psychological stress on humans: Increased production of pro-inflammatory cytokines and TH1-like response in stress-induced anxiety. *Cytokine*, 10(4), pp.313-318.
22. Mazzuoli, Gemma, and Michael Schemann. "Mechanosensitive Enteric Neurons in the Myenteric Plexus of the Mouse Intestine." Ed. Yvette Tache. *PLoS ONE* 7.7 (2012): e39887. *PMC*. Web.

23. National Institute of Diabetes and Digestive and Kidney Diseases. (2018). *Digestive Diseases Statistics for the United States | NIDDK*. [online] Available at: https://www.niddk.nih.gov/health-information/health-statistics/digestive-diseases

24. Nezami, Behtash Ghazi, and Shanthi Srinivasan. “Enteric Nervous System in the Small Intestine: Pathophysiology and Clinical Implications.” *Current gastroenterology reports* 12.5 (2010): 358–365. *PMC*. Web.

25. Rao, Meenakshi, and Michael D. Gershon. “The Bowel and beyond: The Enteric Nervous System in Neurological Disorders.” *Nature reviews. Gastroenterology & hepatology* 13.9 (2016): 517–528. *PMC*. Web.

26. Reintam, Annika et al. “Gastrointestinal Failure in Intensive Care: A Retrospective Clinical Study in Three Different Intensive Care Units in Germany and Estonia.” *BMC Gastroenterology* 6 (2006): 19. *PMC*. Web.

27. Rich A, Leddon SA, Hess SL, Gibbons SJ, Miller S, Xu X, and Farrugia G. Kit-like immunoreactivity in the zebrafish gastrointestinal tract reveals putative ICC Developmental Dynamics 236: 903-911, 2007.

28. Sanders, K. and Koh, S. (2007). Stretch-Activated Conductances in Smooth Muscles. *Mechanosensitive Ion Channels, Part B,* pp.511-540.

29. Scirocco, Annunziata, et al. "Cellular and Molecular Mechanisms of Phenotypic Switch in Gastrointestinal Smooth Muscle." *Journal of Cellular Physiology*, vol. 231, no. 2, Feb. 2016, pp. 295-302.

30. Shah, Eric et al. “Psychological Disorders in Gastrointestinal Disease: Epiphenomenon, Cause or Consequence?” *Annals of Gastroenterology : Quarterly Publication of the Hellenic Society of Gastroenterology* 27.3 (2014): 224–230. Print.

31. Törnblom, H., Sadik, R., Abrahamsson, H. and Simren, M. (2011). Colonic Transit Time and Its Influence on Abnormal Bowel Habits, IBS Symptoms and Psychological Symptoms. *Gastroenterology*, 140(5), pp.S-533-S-534.

32. Tsukamoto, K., Mizutani, M., Yamano, M. and Suzuki, T. (1999). The Relationship between Gastrointestinal Transit and Motility in Dogs with Postoperative Ileus. *Biological & Pharmaceutical Bulletin*, 22(12), pp.1366-1371.

33. Udvadia, A. and Linney, E. (2003). Windows into development: historic, current, and future perspectives on transgenic zebrafish. *Developmental Biology*, 256(1), pp.1-17.

34. Yarandi, S S and S Srinivasan. "Diabetic Gastrointestinal Motility Disorders and the Role of Enteric Nervous System: Current Status and Future Directions." *Neurogastroenterology and Motility: The Official Journal of the European GI Motility Society*, vol. 26, no. 5, May 2014, pp. 611-624

35. Zhou, J, et al. "Human Prokinetic Drugs Promote Gastrointestinal Motility in Zebrafish." *Neurogastroenterology and Motility: The Official Journal of the European Gastrointestinal Motility Society*, vol. 26, no. 4, Apr. 2014, pp. 589-595.