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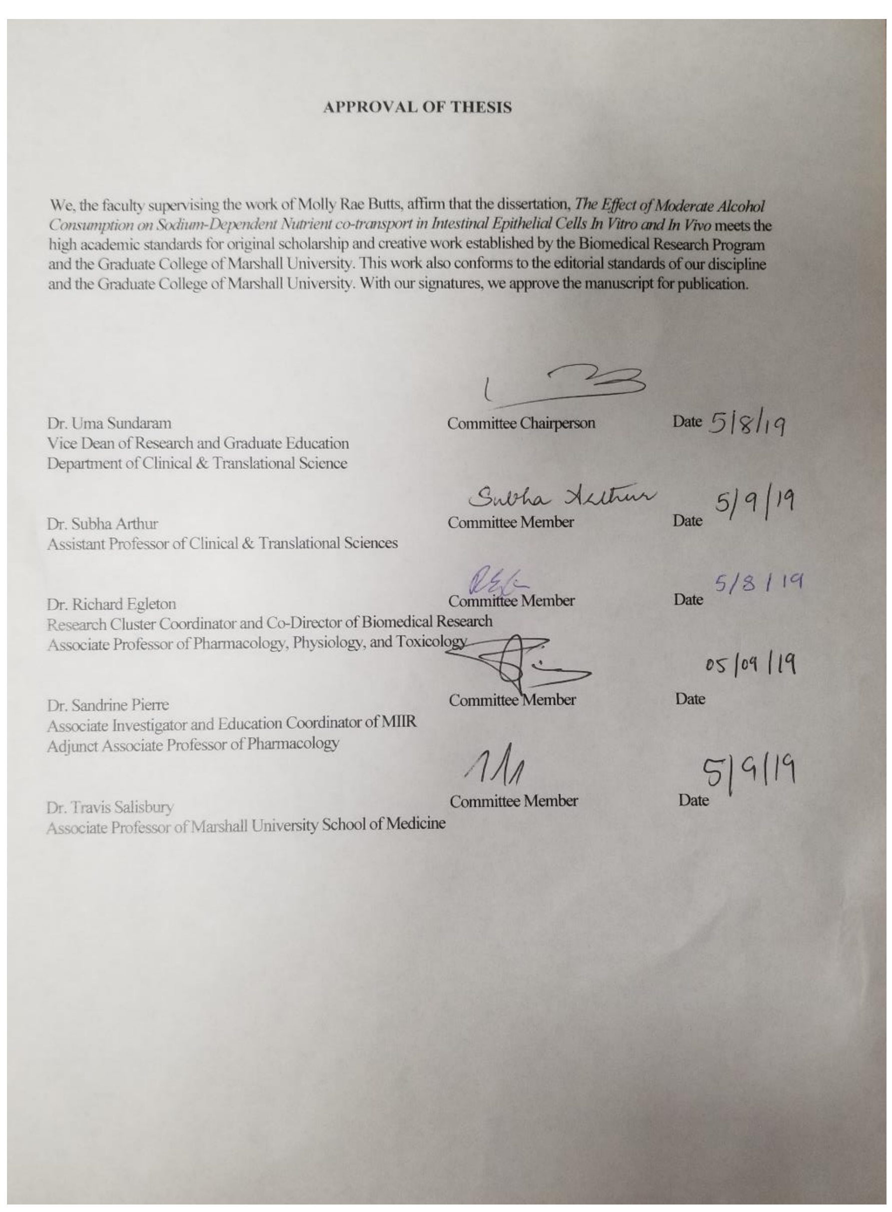
# THE EFFECT OF MODERATE ALCOHOL CONSUMPTION ON SODIUM-DEPENDENT NUTRIENT CO-TRANSPORT IN INTESTINAL EPITHELIAL CELLS *IN VITRO* AND *IN VIVO*

A dissertation submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
In
Biomedical Research
by
Molly Rae Butts
Approved by
Dr. Uma Sundaram, Committee Chairperson
Dr. Subha Arthur
Dr. Richard Egleton
Dr. Sandrine Pierre
Dr. Travis Salisbury

Marshall University
July 2019

**APPROVAL OF THESIS**

We, the faculty supervising the work of Molly Rae Butts, affirm that the dissertation, *The Effect of Moderate Alcohol Consumption on Sodium-Dependent Nutrient co-transport in Intestinal Epithelial Cells In Vitro and In Vivo* meets the high academic standards for original scholarship and creative work established by the Biomedical Research Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.



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Dr. Uma Sundaram — Committee Chairperson — Date 5/8/19
Vice Dean of Research and Graduate Education
Department of Clinical & Translational Science

Dr. Subha Arthur — Committee Member — Date 5/9/19
Assistant Professor of Clinical & Translational Sciences

Dr. Richard Egleton — Committee Member — Date 5/8/19
Research Cluster Coordinator and Co-Director of Biomedical Research
Associate Professor of Pharmacology, Physiology, and Toxicology

Dr. Sandrine Pierre — Committee Member — Date 05/09/19
Associate Investigator and Education Coordinator of MIIR
Adjunct Associate Professor of Pharmacology

Dr. Travis Salisbury — Committee Member — Date 5/9/19
Associate Professor of Marshall University School of Medicine

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## DEDICATION

I dedicate this dissertation to my family.

Valerie Wilkes-Butts and Randall Butts

Megan Butts-Dorler and Randy Butts

Susan Wilkes

Rigley Butts

Will Butts

Gracie Butts

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fluffy muppet who always keeps me walking on the right path. When this dissertation was complete, I purchased him a large green cactus toy for a companion and a bag of his favorite treats, because he deserves them.

In all, I hope that we can augment the bounds of scientific knowledge with this dissertation. I say we, because this dissertation could not have been completed without every one of the aforementioned individuals. My wish is that you know how grateful I am to each one of you for all of your help. Thank you.

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ABSTRACT

**Background:** Alcohol consumption leads to a variety of different health consequences including cardiovascular disease, cancer and malnutrition. This malnutrition is in part due to a sub-optimal diet but also due to the malabsorption of nutrients along the small intestine. Many studies have shown that ethanol directly decreases nutrient absorption along the small intestine; however, few studies have investigated the effect of a moderate dose of ethanol on the transmembrane nutrient co-transporters that line the brush border membrane of the small intestine. The primary fuel source for the small intestine, glutamine, as well as for the entire body, glucose, are absorbed by the sodium-dependent nutrient co-transporters: Na-glutamine co-transport (B0AT1) and Na-glucose co-transport (SGLT1), respectively. Both B0AT1 and SGLT1 are present in the brush border membrane of intestinal absorptive villus, but not crypt cells. The effect of moderate ethanol consumption on B0AT1 and SGLT1 in intestinal epithelial cells is not known. **Methods:** Rat intestinal epithelial cells (IEC-18) were exposed to 8.64 mM ethanol over 1, 3, 6, and 12 hours. Sixteen-week-old Sprague Dawley rats were administered an intragastric gavage of 2 g/kg ethanol over 1, 3, and 6 hours. Sodium-dependent $^{3}$H-glutamine and $^{3}$H-*O*-methyl-*D*-glucose uptakes were conducted to determine B0AT1 and SGLT1 activities, respectively. Na-K-ATPase activity was measured as a function of inorganic phosphate release and with $^{86}$rubidium uptake. Protein expression was analyzed by Western blot analysis and immunohistochemical staining. **Results**: Ethanol significantly decreased glutamine absorption, glucose absorption and Na-K-ATPase activity in enterocytes. Kinetic studies showed a decrease in $V_{max}$ values for B0AT1 *in vitro*, but an increase in $K_m$ values for SGLT1 *in vitro* and *in vivo*. Western blots and immunohistochemistry data support these findings. Ethanol did not change Na-H exchange *in vitro.* Acetaldehyde alone did not change glutamine or glucose uptake *in vitro*. **Conclusions:**

Moderate ethanol significantly inhibits B0AT1 and SGLT1 in intestinal epithelial cells. This occurs through two separate mechanisms: B0AT1 is reduced via a decrease in co-transporter number while SGLT1 is diminished via a decrease in co-transporter affinity for glucose. Moreover, both B0AT1 and SGLT1 are further inhibited at the intact cellular level secondary to the decreased sodium-gradient. Overall, glutamine and glucose absorption by enterocytes is significantly affected by moderate ethanol, which may help describe the onset of alcoholic malnutrition.

# CHAPTER 1

# ETHANOL AND THE SMALL INTESTINE

Many studies have shown that ethanol consumption affects the cellular metabolism, structure and function of the intestine (Bode and Bode 2003). Considering the intestinal epithelium is a sensitive barrier to the outside world, more research should be conducted to fully understand how ethanol affects the cells along the gastrointestinal (GI) tract.

## The small intestine

### Structure and layers of the small intestine

The digestive system breaks down and absorbs all the dietary nutrients essential for the sustenance of life. The human small intestine, where the majority of nutrient absorption occurs, is made up of three segments, the duodenum (10 inches), jejunum (8 feet) and ileum (12 feet). The human small intestine itself is a tube-like structure that has four general layers: starting at the lumen, there is the mucosa, the submucosa, the muscularis externa and the serosa (Figure 1). The mucosa consists of a surface epithelium, lamina propria and muscularis mucosae. The intestinal epithelium is primarily made up of intestinal absorptive cells, called enterocytes, held together by cellular junctions, such as tight junctions (TJ), adherens junctions and gap junctions. While absorbing nutrients, electrolytes and water is the primary function of the intestinal epithelium, it also secretes digestive enzymes, hormones (Marieb 2014) and functions as a barrier to exclude harmful substances such as microorganisms, antigens and toxins from entering the body (Pochini, Scalise et al. 2014). The next layer, the submucosa, consists of connective tissue including blood vessels. The muscularis externa is composed of smooth muscle for GI motility and the serosa is the squamous epithelium layer which reduces friction during GI tract

movement. The layers of the small intestine all work together to form a functioning GI tract (Figure 1) (Marieb 2014).

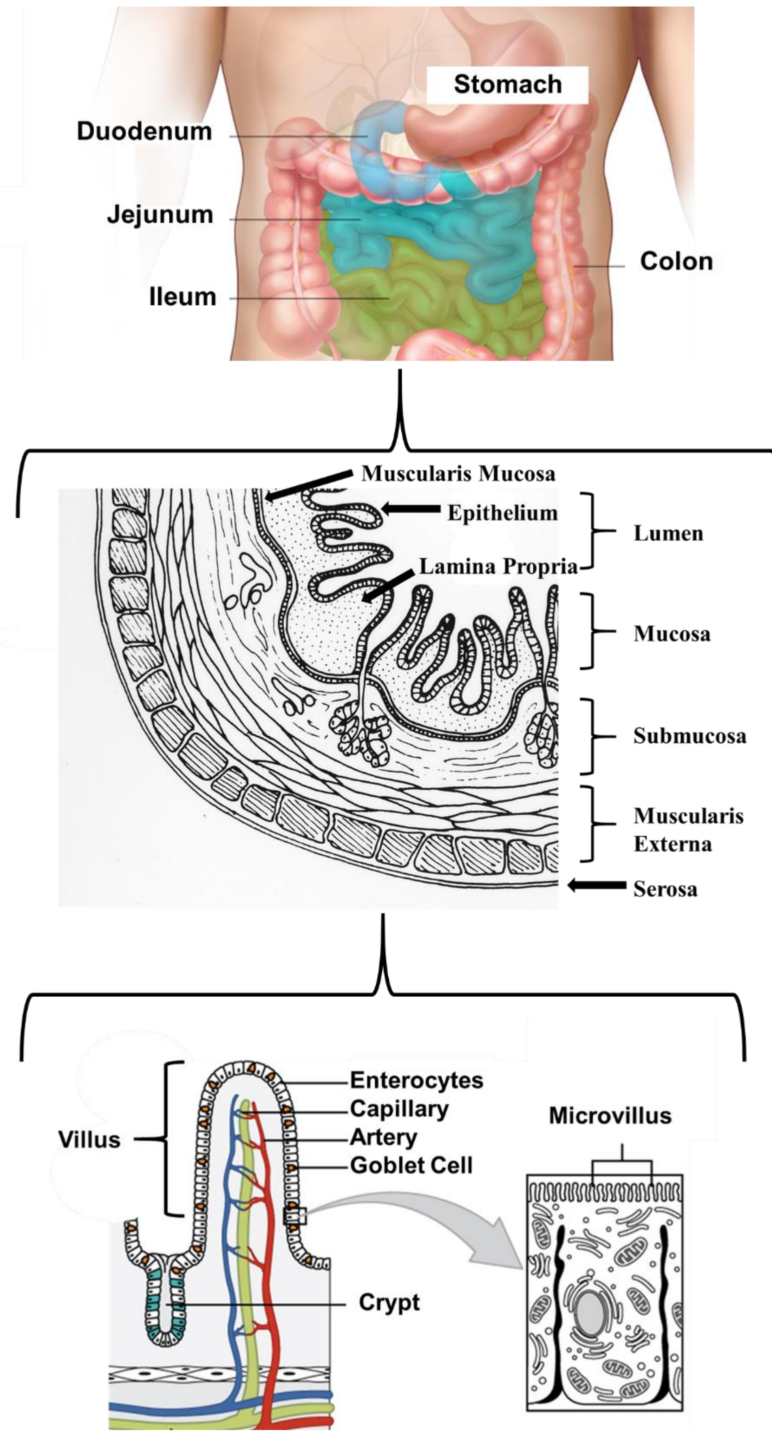


**Figure 1: Physiology of the small intestine.** The small intestine, located in the abdominal cavity, is composed of several layers surrounding the lumen including the mucosa, the submucosa and the serosa. The mucosa contains the intestinal epithelium, which is created from individual intestinal cells. The structure of the small intestine contains mechanisms of increased surface area, including the villi and microvilli. Each image is adapted from the following sources with permission in order (Institute 2001, Campus 2012, Board 2018).

### Cellular architecture of the small intestine

The small intestine has an incredible amount of surface area exposed to the lumen. Although this varies by the individual, the human intestine has a surface area of approximately 32 $m^2$, or approximately half the size of a badminton court (Helander and Fandriks 2014). This is due to a unique intestinal architecture composed of long finger-like projections, called villi, that protrude into the lumen of the small intestine. Individual villi are formed by a single layer of intestinal epithelium composed of many different types of cells including but not limited to absorptive columnar epithelial cells, goblet cells and enteroendocrine cells. Each intestinal absorptive villus cell, or enterocyte, has plasma membrane projections called microvilli that are oriented towards the lumen of the intestine (Marieb 2014). The villi and microvilli increase the surface area of the small intestine by 60 – 120 times! This increases epithelial cell contact with the intestinal lumen, which allows for greater nutrient absorption (Helander and Fandriks 2014). At the base of each villi is a section of proliferative cells located in the connective tissue of the intestine, which is composed of but not limited to Paneth and stem cells, called the intestinal crypt. The crypt contains the differentiating cell population, which continually divides and pushes developing enterocytes up from the crypt renewing the villus section of the intestinal epithelium. Together, the villus and crypt sections of the intestinal epithelium form a functioning intestinal barrier (Shaker and Rubin 2010).

### Brush border membrane (BBM)

Together, microvilli form the BBM on the apical side of the cell, where nutrient absorption occurs. Nutrients are absorbed passively, through cell membranes, and actively, through specific proteins called nutrient co-transporters located at the BBM. The BBM expresses individual nutrient co-transporters which selectively intake essential nutrients like glutamine and

glucose into enterocytes. In the enterocyte, nutrients can quickly gain access to the mesenteric vascular system, which is in close proximity due to the lacteals present in the villi structure. Once in the vascular system, these nutrients can be quickly distributed throughout the body (Marieb 2014).

**Paracellular and transcellular nutrient absorption**

In order to acquire nutrition from the diet, molecules must be absorbed across the intestinal barrier. This movement of nutrients can be transcellular, traveling through the cell, or paracellular, traveling between cells. Paracellular absorption along the small intestine varies, with the duodenum having the greatest amount of paracellular movement and the ileum with the least, based on the cellular junctions between enterocytes. Transcellular absorption is mediated through individual protein nutrient co-transporters and channels which allow for the selective movement of nutrients through the cell membrane of enterocytes, through facilitated transport, secondary active and active transport. These transport processes, in concert, allow for the absorption of all the essential nutrients the mammalian body requires for proper function (Karasov 2017).

**Active transport**

Active transport requires the direct use of energy to move molecules against their concentration gradient, such as through hydrolyzing adenosine triphosphate (ATP). Secondary active transport requires the indirect use of energy by another pump, such as the Na-K-ATPase, to create a favorable sodium gradient across the BBM which a co-transporter can utilize to move a molecule along with sodium. For instance, sodium-dependent nutrient co-transporters require the basolateral Na-K-ATPase to hydrolyze ATP to move three sodium ions out of the cell and two potassium ions into the cell. This creates a low inner sodium gradient in the cell, which the

secondary active co-transporter uses to move nutrients along its concentration gradient and other molecules against their concentration gradient. These gradients can be electrochemically based as well.

### Na-K-ATPase

As previously mentioned, the Na-K-ATPase establishes and maintains intracellular concentrations of sodium and potassium required for secondary active transport processes. The Na-K-ATPase was first discovered in 1957 by Jens C. Skou (Skou 1957). This pump is composed of three main protein subunits: α, β, and FXYD (Kaplan 2002, Clausen, Hilbers et al. 2017). These subunits have different isoform expressions in tissues around the body, including the α1 and β1 subunits in the small intestine (Saha, Manoharan et al. 2015). The α subunit is the catalytic subunit whereas the β1 subunit is primarily for trafficking the pump to the plasma membrane (Kaplan 2002). In addition to its ion pumping function, the Na-K-ATPase has been shown to have a non-enzymatic signaling function specific to the α1 isoform. The binding of the cardiotonic steroid ouabain to the Na-K-ATPase activates the Src kinase at low concentrations, which causes the activation of signaling cascades through protein tyrosine phosphorylation (Tian, Cai et al. 2006). The signaling function has been shown to be critical for cell physiology (Liang, Tian et al. 2007), transport regulation (Cai, Wu et al. 2008), and sodium flux (Liu, Yan et al. 2017). In all, the Na-K-ATPase has important roles in cells, both in maintaining the sodium concentration gradient as well as activating signaling cascades (Clausen, Hilbers et al. 2017).

### Types of nutrient co-transporters

There are specific protein nutrient transporters along the intestine to absorb all the molecules the mammalian body requires. The main transporter families are the ATP binding cassette family, often efflux transporters, and the solute carrier family (SLC), often uptake

transporters. The SLC family will be focused on during this dissertation. There is a huge amount of these transporters, including at least 395 SLC transporters that range in specificity of substrates. Generally, amino acids are primarily absorbed by the SLC3, 6, 7, 25 and 36 families. Carbohydrates like glucose, galactose, and fructose are absorbed by the SLC2, 5 and 37 families. Vitamins are absorbed by SLC19, 25, 46 and 52 whereas metals are absorbed by SLC11, 30, 39 and 40. Polymorphisms in SLC transporters have been identified with various human diseases including inflammatory bowel disease, jaundice, diabetes, and more. More research is required to fully understand the relationship between the transporters of the SLC family and different disease states, such as alcohol-dependent malnutrition (Lin, Yee et al. 2015).

## Alcohol use and associated health consequences

### Prevalence of alcohol use in the United States

Ethanol, commonly known as alcohol, is prevalently used in the United States. According to the 2015 National Survey on Drug Use and Health, 86 percent of the United States population has had alcohol at some point in their lifetime with 56 percent of the population reporting that they consumed an alcoholic beverage in the past month (NIAAA, Gunzerath, Faden et al. 2004). Despite its frequent use, alcohol consumption has a fiscal burden on our society. Alcohol consumption cost the United States an estimated 249 billion dollars in 2010, including but not limited to increased medical care costs. Overall, alcohol use is very prevalent among the population of the United States, but its use has significant fiscal and health consequences on our society.

### Health consequences of alcohol use

Alcohol use is the fifth leading cause of premature death in the world and is responsible for 6 percent of all global deaths as of 2014 (NIAAA). Alcohol use increases risks for a variety

of different diseases including liver disease, high blood pressure, heart disease, stroke, digestive issues, and cancer of the breast, mouth, throat, esophagus, liver and colon (NIAAA, Rehm, Baliunas et al. 2010, Krenz and Korthuis 2012, Roerecke and Rehm 2012, Organization 2018). Furthermore, heavy alcohol use is associated with weight gain and increased obesity risk, as the molecule itself contains 7 kcal/g (Traversy and Chaput 2015). In addition to its health risks, long-term alcohol use also increases risk for societal issues including mental health problems, lost productivity, and alcohol dependence (Organization 2018).

**Definitions of alcohol consumption**

Considering the fiscal, social and health risks associated with alcohol use, it is vital to understand how much alcohol is too much. The National Institute on Alcohol Abuse and Alcoholism defines moderate drinking as one alcoholic beverage a day for women and up to two a day for men, with a standard alcoholic beverage considered the equivalent of 14 g of ethanol (NIAAA, Gunzerath, Faden et al. 2004). One standard alcoholic beverage is equal to 12 fluid ounces of beer, 5 fluid ounces of wine, or 1.5 fluid ounces of an 80-proof liquor. As opposed to moderate alcohol consumption, heavy alcohol use is considered eight or more alcoholic beverages per week for women or 15 or more for men. Binge drinking, another form of heavy alcohol consumption, is considered four or more alcoholic beverages during a single occasion for women or five or more for men (Table 1) (Table 2) (NIAAA, Prevention).

| | | Drinks per Occasion | Drinks per Day | Drinks per Week | Drinks per Month |
|---|---|---|---|---|---|
| **Moderate** | Women | - | 1 | 6 | <24 |
| BAC≤0.04% | Men | | 2 | 7 | <28 |
| **Heavy** | Women | - | >1 | >8 | >32 |
| BAC≥0.04% | Men | | >2 | >15 | >60 |
| **Binge** | Women | ≥4 | - | - | - |
| BAC≥0.08% | Men | ≥5 | | | |

*One drink refers to a standard alcoholic beverage

**Table 1: Levels of alcohol consumption.** One drink refers to one standard alcoholic beverage. BAC – Blood alcohol content (NIAAA, Prevention).

| | Moderate Alcohol Consumption | Heavy Alcohol Consumption |
|---|---|---|
| **BAC (%)** | <0.04 | >0.04 |
| **mg/dL** | <40 | >40 |
| **mM** | <6.84 | >6.84 |
| **v/v (%)** | <0.05 | >0.05 |
| **w/v (g/kg)** | <2 | >2 |

**Table 2: Levels of experimental alcohol consumption.** BAC – Blood alcohol content, v/v – volume by volume, w/v – weight by volume (NIAAA, Prevention).

**Benefits of moderate alcohol use**

Despite the health risks associated with heavy alcohol consumption, there are some health benefits to moderate alcohol use. Moderate alcohol use decreases the risk for heart disease, ischemic stroke (Gunzerath, Faden et al. 2004), diabetes (Yokoyama 2011), and even obesity (Gunzerath, Faden et al. 2004, Stockwell and Room 2012). These health benefits

associated with alcohol consumption stem from any general type of alcoholic beverage (beer, wine or liquor). The decreased risk for heart disease and myocardial infarction stem from beneficial changes to blood lipid profile, clotting factors, vasodilation through increased nitric oxide synthase and more. Moderate alcohol consumption also decreases the risk of type II diabetes and improves insulin sensitivity, by decreasing gluconeogenesis in the liver leading to less glucose leaving the liver and protection for the pancreatic cells (Yokoyama 2011, Krenz and Korthuis 2012).

Interestingly, moderate alcohol use has been linked to a decreased risk for obesity as well. Moderate consumers of alcohol are less likely to gain weight compared to abstainers (Wang, Lee et al. 2010, Traversy and Chaput 2015), which is interesting because ethanol itself contains calories. Furthermore, moderate alcohol use has not been associated with increased weight gain, adiposity, body mass index, or waist circumference (Gunzerath, Faden et al. 2004, Traversy and Chaput 2015). In a study with over 8,000 participants, alcohol use was associated with a lower prevalence of metabolic syndrome (Freiberg, Cabral et al. 2004). The mechanism of this decreased obesity risk is unknown. However, ethanol has been shown to decrease the absorption of nutrients along the small intestine which may lead to the decreased risk of obesity seen with moderate alcohol intake (Bode and Bode 2003).

**Alcohol use and risk of mortality**

The relationship between ethanol and its beneficial health outcomes has been highly debated. Previous studies have determined that the relationship between alcohol use and mortality is J-shaped (Stockwell, Zhao et al. 2016). Initial studies found that those who abstain from alcohol use have a higher risk of mortality than those who moderately drink, leading to the curve in the J-shape. Moreover, those who use alcohol heavily have an increased risk of

mortality, significantly more than those who moderately drink or abstain. However, alcohol intake over longitudinal studies is difficult to define. There are many variables that can alter the overall conclusions drawn. The variables associated with alcohol-dependent longitudinal studies include the dosage of ethanol, frequency of alcohol use, consistent drinking versus episodic drinking, exercise, diet, sex, and many more. A recent study published by Stockwell and colleagues discussed how the decreased risk for mortality based on alcohol use is incorrect; more specifically, the relationship has been inflated due to poorly chosen control groups (Stockwell, Zhao et al. 2016). For example, underlying health issues may force some subjects to abstain from alcohol, creating a group of alcohol abstainers who are more unhealthy than moderate drinkers for reasons other than alcohol consumption. When these unhealthy abstainers are used in mortality studies, their underlying health issues make the control group more likely to become ill, which artificially inflates the benefits of moderate alcohol use. After adjusting for what Stockwell and colleagues determined to be truly comparable abstainers, researchers found that occasional moderate drinking has no net mortality benefit (Stockwell, Zhao et al. 2016). The discrepancies found in these mortality studies are important because they may also be true for the associations between moderate alcohol use and its related health benefits. Clearly, further research is required to understand the relationship between alcohol use and overall health outcomes.

**To drink or not drink, that is the question**

More research needs to be conducted on the possible health benefits or consequences of moderate alcohol use before a more accurate conclusion can be drawn. The previous health benefits attained with moderate alcohol use often have supportive *in vitro* and *in vivo* data and current longitudinal studies are now aware of the possible unhealthy abstainer inflation

associated with these studies. Furthermore, studies have been conducted specifically on moderate alcohol use and the associated decreased risk for cardiovascular disease, which corrected for this unhealthy abstainer phenomenon. Researchers found that there was still a significant decrease in the risk for cardiovascular disease, just not as large of a decreased risk as previously reported (Di Castelnuovo, Costanzo et al. 2006). Does this mean a glass of wine with dinner is safe? Unfortunately, this is still unclear. What is known is that any amount of ethanol can increase cancer risk, even at moderate doses. Overall, it is suggested by the United States Dietary Guidelines to limit alcohol use. The guidelines also suggest abstainers to not start using alcohol. Therefore, the question of whether to consume alcohol or not remains convoluted.

## Gastrointestinal absorption of ethanol

### First pass metabolism of ethanol

Ethanol first interacts with the body through the GI tract. When alcohol is orally consumed, it undergoes initial metabolism in the stomach. Ethanol metabolism requires ethanol to be oxidized to acetaldehyde, ethanol's primary toxic metabolite. This occurs through several pathways. The predominate pathway for ethanol metabolism is through an enzyme called alcohol dehydrogenase (ADH). Isoforms ADH1 and ADH3 are present in the stomach. One of the first papers detailing ethanol absorption along the GI tract was conducted in 1948. Researchers exposed the stomach of Wistar rats to 5 M ethanol for 20 minutes and showed that the stomach was permeable to ethanol (Karel and Fleisher 1948). Therefore, the initial metabolism of ethanol occurs in the stomach, which is referred to as first pass metabolism. First pass metabolism varies based on many factors. Race, age, sex, exercise, previous alcohol use, genetics, biological rhythms, gastric emptying rates and food intake all vary the pace of ethanol metabolism, including first pass metabolism. Therefore, it is important to note that not all ingested ethanol

moves to the vascular system, as some is partially metabolized in the stomach (Cederbaum 2012).

### Ethanol absorption in the small intestine

Ethanol undergoes first pass metabolism in the stomach, but the majority of ingested ethanol is passively absorbed along the upper section of the small intestine called the duodenum. Ethanol passively diffuses along its concentration gradient through cell membranes due to the amphiphilic nature of the molecule. This diffusion rate of ethanol into enterocytes is also determined by a variety of factors including the concentration of ethanol, gastric emptying rate, food intake, dosage, blood flow, intestinal motility, and intestinal wall permeability (Mudie, Amidon et al. 2010, Cederbaum 2012). Nevertheless, the passive diffusion of ethanol is rapid. Ethanol quickly interacts with cell membranes, in the course of nanoseconds (Patra, Salonen et al. 2006). After passing through the cell membranes, causing some alterations in the membrane fluidity of the cell membrane, ethanol can quickly pass into the vascular system where it gains access to the rest of the body. Ethanol is evenly distributed throughout tissues in the body in proportion to their water content, excluding fats (Cederbaum 2012).

### Measuring ethanol in the intestine

Due to ethanol's quick absorption into the vascular system at the duodenum, the jejunum and the ileum of the small intestine are affected by ethanol via the basolateral membrane (BLM) of enterocytes. Unless extremely high dosages of ethanol are consumed, the jejunum and ileum of the small intestine should not be exposed to ethanol via the BBM. Since ethanol affects the ileum through the vasculature, blood alcohol content (BAC) is the most accurate unit of measurement of ethanol for the lower small intestine (Bode and Bode 2003, Krenz and Korthuis 2012). This idea had been well supported by the literature. For example, there was no difference

in gas chromatography-mass spectroscopy analysis and high-performance liquid chromatography of ethanol molality between the ileum and serum of eight-week-old Sprague Dawley rats fed a heavy dose of ethanol (5.42% w/v) for eight weeks (Xie, Zhong et al. 2013). Furthermore, a heavy dose of ethanol (2.75 g/kg) injected intraperitoneally in mice showed mucosal changes. Specifically, villus cells were damaged especially in the ileum of the small intestine (Lee, Choi et al. 2014). This means that ethanol had traveled from the vascular system and into the lumen of the small intestine to cause these disruptions, making BAC the most accurate measurement of ethanol along the lower small intestine (Bode and Bode 2003, Krenz and Korthuis 2012).

## Ethanol's effect on nutrient absorption

### Initial research on ethanol and nutrient absorption

Ethanol's effect on nutrition and its associated research began with a reasonable question: why are many chronic alcoholics malnourished (Williams, Pelton et al. 1955)? This question ignited the initial research between ethanol, nutrition, and the small intestine. The first ethanol and nutrition studies described the effect of ethanol on everted sacs of the rat small intestine, where a binge-like dose of ethanol (3% v/v) inhibited the transport of phenylalanine, leucine, glycine, alanine, methionine, valine and glucose (Chang, Lewis et al. 1967). However, the first breakthrough in the effect of ethanol on a distinct nutrient co-transporter leading to a disease state was conducted by Hoyumpa and colleagues in 1975, when this group established that an intragastric binge-like dose of ethanol (50 to 750 mg/100 g of body weight) negatively affects the active, not passive, transport of low concentrations of thiamine (vitamin $B_1$) (0.06 – 2 μM thiamine) in the everted jejunal segments of rats (Hoyumpa, Breen et al. 1975). These initial studies became one of the first significant papers to make the vital connection between alcohol use, malnutrition and disease states. The thiamine deficiency described in these initial studies is

directly linked to Wernicke-Korsakoff syndrome, a syndrome presenting with an altered mental status including confusion and dementia, ataxia and nystagmus. Although only prevalent in under 2% of the population, Wernicke-Korsakoff syndrome is more common in chronic alcohol abusers, malnourished individuals, and at one point, babies whose formula was thiamine deficient (S Akhouri 2019). The brain atrophy associated with this syndrome is directly caused by thiamine deficiency, which stems from the effect of ethanol on the thiamine transporter in chronic alcoholics (Thomson 2000, S Akhouri 2019).

Further research by this group led to a long-term study of a heavy dose of ethanol using a Lieber DeCarli liquid diet (36% ethanol). The diet brought the BAC of rats to over 0.08% for six to eight weeks. Interestingly, researchers found that Na-K-ATPase activity and thiamine transport were unchanged in this long-term study. However, this long-term ethanol diet, in addition to a binge-like dose of ethanol using an intragastric gavage (250 mg/100 g), leading to BAC of above 0.19%, decreased serosal thiamine transport and Na-K-ATPase activity. Overall, researchers determined that acute systemic ethanol may be more important than duration of exposure regarding thiamine transport (Hoyumpa 1980). At this point, the field of ethanol and nutrition was fully introduced and now more than fifty years of ethanol and nutrition research exists, from which the relevant findings are described in the following section.

**Ethanol's effect on carbohydrate, protein and lipid absorption**

Chronic alcoholics are commonly malnourished, which may stem from a decrease in caloric intake, but also from an ethanol-mediated decrease in nutrient absorption at the small intestine (Bode and Bode 2003). The net absorption of a nutrient solution was analyzed in chronic alcoholics, without alcohol-dependent malnutrition or other intestinal dysfunctions, using an intestinal perfusion technique (Pfeiffer, Schmidt et al. 1992). Researchers found that

chronic ethanol decreased the net absorption of water, carbohydrates, proteins and lipids in the duodenum, but not the upper jejunum. The duodenum may be more susceptible to nutrient malabsorption due to increased cellular damage from exposure to higher concentrations of ethanol present only in the duodenum (Pfeiffer, Schmidt et al. 1992, Bode and Bode 2003).

**Ethanol and lipid absorption**

Chronic ethanol had biphasic effects on lipid absorption in the small intestine. A moderate dose of ethanol, 0.75 g/kg over one hour, increased the transport of dietary fat but a heavy dose of ethanol, 36% ethanol liquid diet for 3 to 4 weeks, inhibited the absorption of dietary fats in rats (Baraona and Lieber 1975). Similarly, the absorption of arachidonic acid and linoleic acid was decreased in human jejunal tissue exposed to a heavy dose of ethanol (100 mM) (Barros, Chen et al. 1990). Decreased fatty acid absorption was also found in the duodenum and the jejunum of chronic alcoholics (Pfeiffer, Schmidt et al. 1993). Further research is required to better understand the mechanism behind the altered absorption of lipids in the small intestine following ethanol exposure.

***D*-glucose**

Chronic ethanol decreases absorption of glucose in *in vivo* models as well as in humans (Bode and Bode 2003). The effect of ethanol on sodium-dependent glucose co-transport was investigated in the jejunum of hamsters (Dinda, Beck et al. 1975). Researchers showed that a binge-like dose of ethanol (450 mM) decreased intracellular water and intracellular glucose, suggesting an inhibition of active transport. These researchers determined that the net flux of sodium did not significantly decrease, suggesting that there may be an additional interference of the carrier-mediated coupled entrance of glucose and sodium across the BBM. The researchers went further to describe the effect of ethanol on the serosal and luminal side of the hamsters in

another study. Researchers found that ethanol on the serosal side had no effect on glucose and water absorption (Dinda and Beck 1977). Further studies were conducted using jejunal purified BBM vesicles (BBMV) treated with a binge-like dose of ethanol (1-5% w/v) in hamsters as well (Dinda and Beck 1981). Ethanol was directly added to the BBMV, which then showed a decrease in glucose absorption. This process was reversible with several ethanol-free washes. Overall, these researchers showed that the effect of ethanol on glucose absorption is at the level of the BBM (Dinda and Beck 1981).

Moreover, ethanol inhibited glucose absorption in the small intestine of rats using a binge-like dose of 3% ethanol in a perfusion study (Cobb, Van Thiel et al. 1983). This phenomenon was also investigated in the jejunum of chickens (Yunus, Awad et al. 2011). Using Ussing chambers, a moderate dose of ethanol decreased active glucose absorption in the intestinal epithelia (0.33% and 0.66%) (Yunus, Awad et al. 2011). A binge-like dose of ethanol at 3.5% and 7% in canine jejunal Thirty-Vella loops demonstrated a net decrease of glucose absorption by 13% and 26% respectively (Money, Petroianu et al. 1990). Serum glucose levels were unaffected (Money, Petroianu et al. 1990).

Researchers investigated further into the effect of ethanol on glucose absorption at the BBM. Sprague Dawley rats treated with a heavy dose of ethanol (5% v/v) for 28-32 days increased the absorption of glucose, whereas incubation of the vesicles with binge-like dose of ethanol (4% v/v) reduced the absorption of glucose in prepared BBMVs from the jejunum (al-Balool and Debnam 1989). Overall, researchers hypothesized that the stimulation of glucose in the chronic model may be due to a compensatory mechanism (al-Balool and Debnam 1989). Kinetic studies using rats exposed to chronic ethanol examined the effect on glucose absorption. This study reported that ethanol reduced the maximal rate of uptake of glucose absorption in

Wistar rats fed a heavy dose of ethanol (20% v/v) by Ryle's tube for 40 days via a decrease in $V_{max}$, not $K_m$ (Kaur, Kaur et al. 1995).

Overall, the inhibitory effect of ethanol on sodium-dependent glucose co-transport was determined in the intestine of hamsters (Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981), rats (Cobb, Van Thiel et al. 1983, al-Balool and Debnam 1989, Kaur, Kaur et al. 1995), chickens (Yunus, Awad et al. 2011), dogs (Money, Petroianu et al. 1990) and humans (Bode and Bode 2003). Collectively, researchers were able to establish that ethanol affects glucose absorption at the BBM of the small intestine.

***D*-xylose**

Ethanol either decreases or does not affect xylose absorption in animal and human models (Bode and Bode 2003). Further research is necessary.

***L*-leucine**

The effect of ethanol on leucine absorption is only present in acutely-treated rats (Bode and Bode 2003). For example, intestinal absorption of leucine was not inhibited following a heavy dose of ethanol (20% v/v) in Sprague Dawley rats for 7 weeks (Hajjar, Tomicic et al. 1981) or during 40 days of the same volume of ethanol administered via Ryle's tube in Wistar rats (Kaur, Kaur et al. 1995). In another study, four weeks of ethanol did not significantly change total leucine absorption in the rat small intestine, but ethanol did have differing segmental effects on the upper jejunum and lower ileum. Specifically, the active uptake of leucine was increased in the jejunum compared to the ileum in response to ethanol in these rats, but it was not statistically significant (Martines, Morris et al. 1989). Finally, chronic ethanol did not produce a statistically significant change in leucine absorption at varying concentrations of leucine using a multiple-pass perfusion technique in rats (Carreras, Vazquez et al. 1993). This study showed that chronic

ethanol dosages, as opposed to acute ethanol dosages, can lead to varying effects of ethanol on nutrient absorption.

#### ***L*-glycine and *L*-alanine**

In *Xenopus llaevis* oocytes treated with a binge-like dose of 200 mM ethanol for three minutes, ethanol reduced dipeptide transport via the hPepT1 (*SLC15A1*) transporter (Li, Xu et al. 2008). hPepT1 mainly transports di and tripeptides, but ethanol reduced glycyl-sarcosine currents by 42% while reducing alanyl-alanine currents by 30% without affecting the affinity of the co-transporter ($K_m$) (Li, Xu et al. 2008). Furthermore, sodium-dependent glycine absorption was significantly decreased in Wistar rats administered a heavy 20% v/v dose of ethanol for 40 days (Kaur, Kaur et al. 1995). Overall, researchers demonstrated that ethanol can also directly affect the transport of peptides in the small intestine as well.

### **Ethanol's effect on vitamin absorption**

#### **Thiamine (vitamin $B_1$)**

Alcohol-dependent thiamine deficiency can lead to Wernicke-Korsakoff syndrome (Thomson 2000). As previously discussed, ethanol significantly decreases the active transport of low concentrations of thiamine in the small intestine of rats exposed to chronic ethanol (Hoyumpa, Breen et al. 1975, Hoyumpa 1980, Bode and Bode 2003). Heavy ethanol administration for 2, 4, and 6 weeks in male Wistar rats also showed decreased thiamine transport along the jejunal BBM and BLM at the level of the thiamine transporter. Ethanol reduced the protein and mRNA expression of thiamin transporter-1, but not thiamine transporter-2. Researchers were able to show a corresponding reduction in the thiamine transporter's transcriptional promotor activity levels. Furthermore, this phenomenon of decreased thiamine

transport was also reproducible in human intestinal epithelial HuTu-80 cells (Subramanya, Subramanian et al. 2010).

Interestingly, there may be some differences in types of alcohol consumed and the associated effect of ethanol on thiamine transport specifically. In a study conducted in the jejunum of male Wistar rats, a binge-like dose of 12% v/v ethanol and wine both reduced $^{3}$H-thiamine mucosal-to-serosal permeability by approximately 60% using Ussing chambers, but in a 21-day treatment, red wine alone did not cause a decrease in thiamine permeability. Therefore, the type of ethanol tested in these nutrition studies is important to consider (Lemos, Azevedo et al. 2005). Overall, ethanol-mediated thiamine deficiency occurs at the level of the thiamine transporter and can be reproduced in humans which is important due to its direct causation of Wernicke-Korsakoff syndrome (Thomson 2000, S Akhouri 2019).

**Folate (vitamin $B_9$)**

Folate deficiency during pregnancy can lead to developmental deformities including in the neural tube of fetuses. In humans, folate deficiency is present in approximately two thirds of chronic alcoholics which is in part due to folate deficiency in the diet, but also due to decreased absorption of folate in response to ethanol (Bode and Bode 2003). One-hour treatment of a binge-like dose of ethanol (65 mmol/L) decreased apical folate absorption in renal human proximal tubule cells by 20-25%. This ethanol-mediated decrease in folate absorption was not seen at the BLM.

Furthermore, increased expression of the reduced folate carrier and folate receptor occurred in response to 14 days of 5% w/v ethanol exposure in the nephrons of rats. This study used a dose of ethanol researchers deemed ‘sub-chronic,’ less than 65 mmol/L, in which researchers determined that the longer exposure to lower levels of ethanol increased the

expression of the folate carrier and folate receptor, but acute doses led to inhibitory effects on folate transport (Romanoff, Ross et al. 2007). However, a 20% dose of ethanol showed no change in folate transport in jejunal everted sacs in rats (Racusen and Krawitt 1977). Additionally, a previously discussed study using a heavy 21-day dose of 12% v/v red wine or ethanol in male Wistar rats found no change in folate transport in the jejunum as well (Lemos, Azevedo et al. 2005). Therefore, changes to folate absorption were seen in the kidney at the level of the folate carrier and receptor in response to ethanol (Romanoff, Ross et al. 2007), but some studies did not see changes in intestinal absorption of folate in response to ethanol (Racusen and Krawitt 1977, Lemos, Azevedo et al. 2005).

Other studies conducted more specifically at the BBM of the small intestine have shown malabsorption of folate upon exposure to ethanol (Hamid, Wani et al. 2007, Thakur, Rahat et al. 2015). Researchers found that male Wistar rats fed a moderate 1 g/kg dose of ethanol for three months showed decreased $^{3}$H-folate transport at the intestinal BBM. Kinetic studies presented decreased $V_{max}$ and increased $K_m$ values, suggesting both the number of folate transporters and the affinity of the folate transporters were decreased in response to ethanol. This decrease in folate absorption in response to ethanol was shown throughout the crypt-villus axis. At the level of the folate carrier, protein and mRNA expression levels were decreased in the jejunal tissue along the entire crypt-villus axis as well. Overall, researchers determined that this decrease in folate absorption was not due to a change in the architecture of the intestine, as this phenomenon was observed throughout the crypt-villus axis, but because a change in localization of the folate carrier from the BBM occurred (Hamid, Wani et al. 2007).

Furthermore, this effect was shown to be reversible in rats treated with a heavy dose of ethanol as above for three months, and then given a two-month reprieve (Thakur, Rahat et al.

2015). In this additional study, it was suggested that reduced binding of the SP1 transcription factor in the promotor regions of the folate carrier may be the cause of the decrease in folate carrier in response to ethanol (Thakur, Rahat et al. 2015).

Overall, it is important to note that in the past, experiments conducted with certain procedures may not have found changes in the absorption of certain nutrients based on dosage, type of ethanol (beer, wine, or liquor), and even the experimental techniques used. These variables may explain why decreased absorption of folate was not found in past studies (Racusen and Krawitt 1977, Lemos, Azevedo et al. 2005). Nevertheless, ethanol clearly has an effect on folate absorption in the kidney and small intestine at the level of the folate transporter (Bode and Bode 2003, Hamid, Wani et al. 2007, Romanoff, Ross et al. 2007, Thakur, Rahat et al. 2015).

**Riboflavin (vitamin $B_2$)**

Riboflavin absorption was inhibited in male Wistar rats fed the Lieber DeCarli diet for four weeks at the BBM and BLM of the small intestine (Subramanian, Subramanya et al. 2013). Decreased riboflavin transporter protein expression and hnRNA levels, which measures rate of transcription, were present. Ethanol-mediated riboflavin malabsorption was also demonstrated in the colon and the kidney. In the kidney, renal BBM and BLM absorption was reduced as well as the protein, mRNA, and hnRNA expression levels of the riboflavin transporter. Overall, this study presents another case in which ethanol mediates a decrease in the nutrient absorption along the small intestine, as well as in other organs (Subramanian, Subramanya et al. 2013).

**Vitamin C**

In chronic alcoholics, low blood levels of ascorbic acid, a fraction of vitamin C, are often present (Bode and Bode 2003). In addition to excess excretion and dietary insufficiencies present in alcohol-dependent malnutrition, ethanol has been shown to affect the vitamin C co-

transporters directly. In Kunming mice fed a heavy dose of 25% ethanol diet for two weeks, ethanol increased the expression of two sodium-dependent vitamin C co-transporters in the kidney and the intestine (Guo, Wang et al. 2013). The increased expression of these vitamin C co-transporters was reversed with the addition of 1.5 mM or 3 mM vitamin C supplementation, which suggests that vitamin C can regulate itself, by suppressing hypoxia-inducible factor-α (Guo, Wang et al. 2013). This study stands out, as it provides evidence that ethanol does not suppress expression of all nutrient transporters.

#### Other vitamins

Vitamins A, D, E, and K are mildly deficient in chronic alcoholics, which varies by the individual (Bode and Bode 2003). The exact mechanism of the malabsorption of these other vitamins is yet to be explored. Moreover, vitamin $B_6$ (pyridoxal-5-phosphate) deficiency is frequently observed in chronic alcoholics (Bode and Bode 2003) which is in part due to ethanol-dependent inhibition of intestinal hydrolysis of the vitamin (Middleton 1986).

### Ethanol's effect on mineral absorption

#### Calcium

Net calcium absorption was decreased following an intragastric gavage of moderate ethanol (2 g/kg) in fasted rats (Krishnamra and Limlomwongse 1987). Ethanol stimulated calcium secretion in the stomach and the distal small intestine, while inhibiting absorption along the proximal small intestine. Furthermore, the addition of heavy dose of 7% ethanol, but not 3.5% ethanol, in canine jejunal Thirty-Vella loops increased the secretion of calcium. Serum calcium levels were unaffected (Money, Petroianu et al. 1990). An acute 3 g/kg intragastric gavage of ethanol also led to net calcium secretion after 30 minutes in rats (Krishnamra and Limlomwongse 1987). Overall, ethanol stimulates calcium secretion and decreases calcium

absorption. However, it is debated on how much ethanol affects net calcium absorption and secretion in the entire small intestine.

### Zinc

Zinc deficiency is associated with chronic alcohol use (Bode and Bode 2003). Male Sprague-Dawley rats fed a Lieber DeCarli ethanol diet for one month displayed decreased zinc absorption (16%) in the ileum using *in vivo* perfusion (Antonson and Vanderhoof 1983). Furthermore, male Sprague Dawley rats fed a Lieber DeCarli diet for six weeks showed that the zinc transporter mRNA expression levels were significantly decreased at both the apical and BLM of the duodenum (Joshi, Mehta et al. 2009). Zinc absorption also plays a significant role in ethanol-mediated TJ disruption discussed later.

### Iron

Higher concentrations of iron are common in chronic alcoholics. In six male chronic alcoholics, intestinal iron absorption was increased two-fold (Duane, Raja et al. 1992). Michaelis-Menten kinetic experiments in tissues from control subjects and chronic alcoholics showed a linear relationship with exposure to increasing iron concentrations. Further experiments showed an increase in intestinal permeability as well, and it was hypothesized that this increase in iron absorption in chronic alcoholics is due to an increased paracellular route of passive absorption of iron in chronic alcoholics (Duane, Raja et al. 1992). In another study, a heavy dose of ethanol was administered in the drinking water of male Swiss mice (200 mL/L) over 13 weeks (Sabino, Petroianu et al. 2010). It was determined that iron absorption in the duodenum was not altered. Therefore, ethanol increases the paracellular passive absorption of iron in the lower small intestine alone (Sabino, Petroianu et al. 2010).

### Magnesium

Hypomagnesaemia is common in chronic alcoholics, but it is due to excess excretion rather than due to malabsorption of magnesium (Bode and Bode 2003).

### Selenium

Diminished selenium is present in the serum of chronic alcoholics (Bode and Bode 2003). However, duodenal absorption of selenomethionine increased in Wistar rat offspring through an increase in the affinity of the transporter ($K_m$) following a heavy dose of 20% v/v ethanol dose during the four weeks of gestation and lactation (Nogales, Ojeda et al. 2011). This study describes a selective case in which the absorption of a nutrient increased in response to ethanol. The differences in selenium presence, between those reported in the serum of chronic alcoholics and this study, may be due to the age of the rats used in this study (Nogales, Ojeda et al. 2011).

### Summation of ethanol's effect on nutrient absorption

The effect of ethanol on nutrient absorption has been well-detailed by a vast amount of literature spanning the mid-20$^{th}$ century. Ethanol decreases the absorption of a wide variety of nutrients including vitamins and glucose (Table 3). This malabsorption stems from dietary deficiencies in chronic alcoholics, but at the cellular level, is also due to changes to nutrient absorption by nutrient transporters. Throughout the research, this phenomenon spans many species and tissues, but each nutrient is differentially affected by ethanol. Further research is required to tease out the action of ethanol on each affected nutrient. In fact, ethanol may affect other nutrients not yet investigated (Bode and Bode 2003). One nutrient that has not yet been examined under ethanol exposure is the conditionally essential amino acid glutamine, which will be discussed in the following chapters.

| Nutrient | Effect on Absorption | Dosage | Location | Major Associated Health Issues | References |
|---|---|---|---|---|---|
| **Thiamine** | ↓ or n/c* | Heavy/Binge >0.08% BAC | Jejunum HuTu-80 cells | Wernicke-Korsakoff syndrome | (Hoyumpa, Breen et al. 1975, Hoyumpa 1980, Hoyumpa, Patwardhan et al. 1981,Subramanya, Subramanian et al. 2010,Lemos, Azevedo et al. 2005) |
| **Riboflavin** | ↓ | Heavy >0.08% BAC | Jejunum Colon | Hyperemia, edema, skin disorders… | (Subramanian, Subramanya et al. 2013) |
| **Folate** | ↓, n/c* | Heavy/Binge >0.08% BAC | Jejunum | Developmental issues, anemia | (Racusen and Krawitt 1977,Hamid, Wani et al. 2007,Thakur, Rahat et al. 2015) |
| **Glucose** | ↓ | Heavy/Binge >0.08% BAC | Jejunum | Malnutrition | (Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981,Cobb, Van Thiel et al. 1983,Money, Petroianu et al. 1990,Yunus, Awad et al. 2011,al-Balool and Debnam 1989,Kaur, Kaur et al. 1995) |
| **Leucine** | ↓ or n/c | n/c in heavy >0.08 BAC | Jejunum | Lethargy, developmental issues, weight loss… | (Hajjar, Tomicic et al. 1981,Kaur, Kaur et al. 1995,Martines, Morris et al. 1989,Carreras, Vazquez et al. 1993) |
| **Vitamin C** | ↑ | Heavy >0.08% BAC | Whole small intestine | Scurvy | (Guo, Wang et al. 2013) |
| **Vitamins A, D, E, $B_6$ and K** | ↓ | Heavy >0.08% BAC | - | Blindness, inability to clot, rickets… | (Bode and Bode 2003) |
| **Calcium** | ↓ | Moderate <0.04% BAC | Duodenum | Osteoporosis | (Krishnamra and Limlomwongse 1987) |
| **Zinc** | ↓ | Heavy >0.08% BAC | | Delayed growth, impotence | |
| **Iron** | ↑ in ileum n/c in duodenum | Heavy >0.08% BAC | Whole small intestine | Anemia | (Duane, Raja et al. 1992,Sabino, Petroianu et al. 2010) |
| **Selenium** | ↑ | Heavy >0.08% BAC | Duodenum | Keshin-Beck and Keshan disease | (Nogales, Ojeda et al. 2011) |
| **Amino Acids** | ↓ | Binge 3% v/v | Whole small intestine | - | (Chang, Lewis et al. 1967) |
| **Proteins** | ↓, n/c** | Heavy >0.08% BAC | Duodenum | - | (Pfeiffer, Schmidt et al. 1992, Pfeiffer, Schmidt et al. 1993) |
| **Lipids** | ↓, n/c** | Heavy >0.08% BAC | Duodenum | - | (Pfeiffer, Schmidt et al. 1992, Pfeiffer, Schmidt et al. 1993,Barros, Chen et al. 1990,Baraona and Lieber 1975) |

**Table 3: Ethanol's effect on intestinal nutrient absorption.** * - n/c only refers to Lemos, Azevedo et al. 2005, ** - n/c only present in upper jejunum, n/c – no change, ↓ - decrease, ↑ - increase, BAC – blood alcohol content.

## ETHANOL METABOLISM

Ethanol clearly affects nutrient absorption along the small intestine. However, how ethanol affects each nutrient is not clear. There must be mechanisms of action of ethanol on intestinal cells, in order to have such a pervasive effect on nutrient absorption. To better explore potential mechanisms that describe how ethanol affects nutrient absorption, the effect of ethanol in cells must be fully examined, starting with the metabolism of ethanol.

### Ethanol metabolism via ADH

Ethanol, as previously mentioned, is oxidized to acetaldehyde through ADH. This enzymatic reaction is reversible. Acetaldehyde is toxic at extremely low concentrations, forming adducts at concentrations as low as 5 μM; therefore, the body quickly oxidizes acetaldehyde to its non-harmful metabolite acetate using aldehyde dehydrogenases (ALDH) primarily in the liver (Fisher, Swaan et al. 2010). As ethanol is oxidized, nicotinamide adenine dinucleotide (NAD) accepts the hydrogen atoms removed from ethanol and then acetaldehyde, which changes NAD to NAD+ to NADH. In cells, this lowers the NAD+/NADH redox ratio which can inhibit glycolysis, the citric acid cycle, pyruvate dehydrogenase, fatty acid oxidation and gluconeogenesis. The acetate produced from the metabolism of ethanol is used to form acetyl coenzyme A, which is an important precursor in all major nutrients like fat, carbohydrates and protein (Figure 2) (Cederbaum 2012).

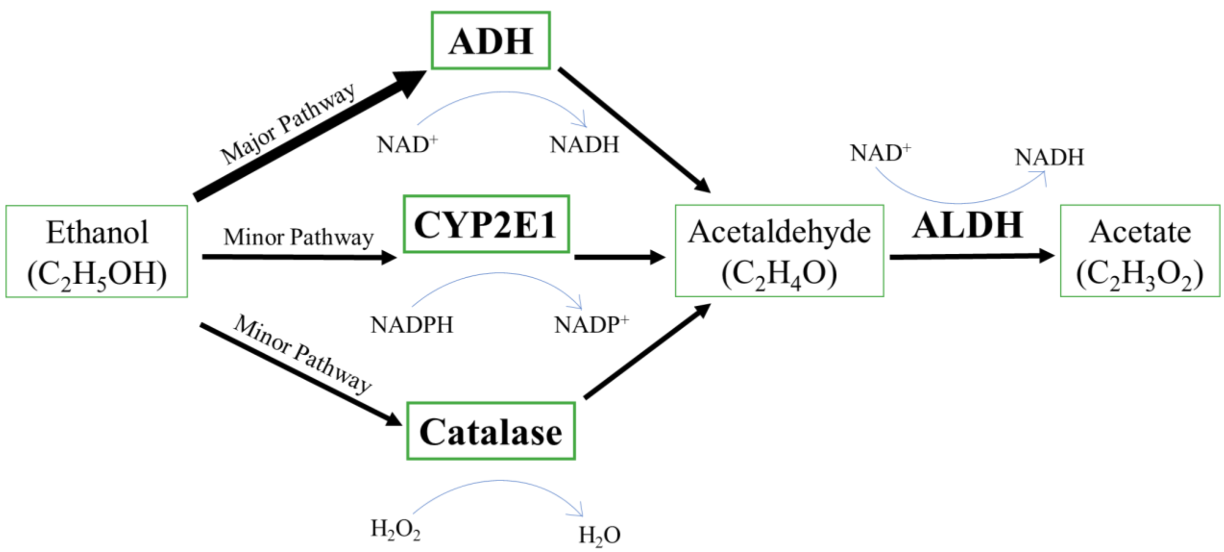


**Figure 2: Pathways of ethanol metabolism.** Ethanol is mainly metabolized by alcohol dehydrogenase in the liver creating acetaldehyde. Other pathways of ethanol metabolism exist, primarily using the CYP2E1 and catalase enzymes. Acetaldehyde is then rapidly metabolized to acetate through alcohol dehydrogenase. ADH – alcohol dehydrogenase, ALDH – Aldehyde dehydrogenase, NAD+ - nicotinamide adenine dinucleotide (oxidized), NADH - nicotinamide adenine dinucleotide (reduced), CYP2E1 – cytochrome P450 2E1.

**Alcohol dehydrogenase**

Ethanol is primarily metabolized through the enzyme ADH. ADH has five isoenzymes in many tissues including in the cytosolic fractions of liver, intestinal (Julia, Farres et al. 1987, Bode and Bode 2003, Fisher, Swaan et al. 2010), kidney, testicular and uterine cells. In the intestine, ADH2 is the predominate isoform (Julia, Farres et al. 1987). ADH broadly oxidizes primary and secondary alcohols. Although this one type of enzyme is the predominant machinery responsible for ethanol metabolism, this enzymatic reaction is not a zero-order process; therefore, ethanol is not metabolized at a constant rate. The kinetic ability of ADH isoenzymes is approximately 1 mM, which is low and slow. Therefore, at higher concentrations of ethanol, ADH becomes easily saturated. As a compensatory mechanism, alternate pathways of ethanol

metabolism become more active: including cytochrome P450 2E1 (CYP2E1) discussed below (Cederbaum 2012).

**Alternate pathways of ethanol metabolism**

The metabolism of ethanol can occur through several different pathways: primarily through ADH, and to a lesser extent through peroxisomes and the cytochrome P450-dependent ethanol-oxidizing system. Ethanol can also interact with fatty acid ethyl esters as a form of metabolism as well (Cederbaum 2012).

**Peroxisomes**

Ethanol oxidation in peroxisomes occurs through an enzyme called catalase. Catalase can oxidize ethanol to acetaldehyde using peroxide but has a slower metabolism rate of ethanol due to the lower levels of peroxide in cells (Cederbaum 2012).

**CYP2E1**

Ethanol is also metabolized through the microsomal cytochrome P450 enzyme, mainly in the liver. P450s are found in the mitochondria and endoplasmic reticulum. There are many isoforms of P450s; moreover, CYP2E1 is a type of P450 enzyme which metabolizes ethanol. The $K_m$ of CYP2E1 is 10 mM, which is higher than the $K_m$ for ADH. However, at low concentrations of ethanol, CYP2E1 may only account for 10% of total ethanol metabolism. On the other hand, at higher concentrations, the CYP2E1 plays a greater role in ethanol metabolism and expresses increased function during chronic ethanol administration. CYP2E1 degrades ethanol at higher rates than ADH, which surprisingly leads to an even higher overall metabolism rate of ethanol at higher ethanol concentrations which plays a role in ethanol tolerance (Cederbaum 2012).

## Acetaldehyde metabolism

### Aldehyde dehydrogenase

ALDH oxidizes acetaldehyde to acetate rapidly. ALDH is present in the mitochondrial, microsomal and cytosolic compartments which have various isoforms of ALDH with differing $K_m$ values. Overall, ALDH is highly present in the liver, and the enzyme is rarely saturated. This enzyme is very efficient because accumulation of acetaldehyde can lead to nausea, vomiting, increased heart rate and other negative symptoms (Cederbaum 2012). Therefore, it is important to fully express this enzyme involved in ethanol metabolism. However, ALDH expression can vary by race, with a significant amount of people of East Asian descent having an inactive form of ALDH2 leading to higher concentrations of acetaldehyde in the blood. This phenotype leads to increased vasodilation and nausea when consuming alcohol in these individuals (Cederbaum 2012). This inactive form of ALDH2 also increases cancer risk along the GI tract in these individuals (Seitz and Stickel 2010).

### Consequences of acetaldehyde

#### Concentration of acetaldehyde in the gastrointestinal tract

Acetaldehyde studies often take place in the colon, rather than in the small intestine, since acetaldehyde concentrations in the colon often exceed concentrations present in the small intestine which is due to the low rate of acetaldehyde metabolism in the colon coupled with the presence of colonic bacteria that also produce acetaldehyde from endogenous and ingested alcohols (Basuroy, Sheth et al. 2005). Researchers have determined that the concentration of acetaldehyde can reach 0.5 to 3 mM in the colon following a dose of 1.5 g/kg ethanol in rats which is an impressive concentration as much smaller concentrations of acetaldehyde (99-760 μM) can lead to increased permeability in Caco-2 cells, a colon cancer-derived cell line

(Atkinson and Rao 2001). In the intestine, concentrations of acetaldehyde can reach upwards of 100 µM (Jokelainen, Matysiak-Budnik et al. 1996, Koivisto, Kaihovaara et al. 1999, Ferrier, Berard et al. 2006).

**Acetaldehyde adducts**

When acetaldehyde is not rapidly metabolized, this molecule can cause havoc around the cell. Acetaldehyde, at extremely low concentrations, can react with thiols and amino groups to form acetaldehyde adducts that directly cause cellular toxicity (Cederbaum 2012). Acetaldehyde can bind to DNA and form carcinogenic adducts and create genetic polymorphisms (Seitz and Stickel 2010). These adducts can form at concentrations as low as 5 µM of acetaldehyde. Lysine is the most common adduct formed with acetaldehyde, which is called a Schiff's base (Fisher, Swaan et al. 2010). Acetaldehyde can also increase tumorigenesis. In a study using ALDH1B1 knock down mice, a heavy dose of ethanol (20% v/v) for one year led to high concentrations of plasma acetaldehyde (1.5 µg/mL) (Muller, Zhou et al. 2017). All ethanol-exposed mice had intestinal hyperproliferation and advanced intestinal tumors with no increase in tumor incidence which suggests that excess acetaldehyde increases the proliferation of tumors in these mice. Overall, acetaldehyde must be rapidly metabolized in cells to avoid causing cellular damage and even tumorigenesis (Muller, Zhou et al. 2017).

**Acetaldehyde's action on TJs**

One of acetaldehyde's major actions on the intestine occurs through impairing the intestinal epithelial barrier by increasing TJ permeability (Bode and Bode 2003, Fisher, Swaan et al. 2010). In Caco-2 cells treated with a heavy dose of ethanol (20% v/v), leading to acetaldehyde concentrations of approximately 1 mM, permeability of low weight molecular markers, mannitol and sucrose, were increased. The same phenomenon was able to be shown

with rats treated with heavy dose of ethanol (5 g/kg) for six days. However, acetaldehyde had no change in permeability of the high weight molecular marker inulin in these experiments (Fisher, Swaan et al. 2010).

In another study investigating acetaldehyde's effect on TJ permeability in Caco-2 cells, researchers determined that genistein (30-300 μM) was able to reverse the decreased permeability in TJs in response to 650 μM acetaldehyde. Researchers also began to illustrate a mechanism of how acetaldehyde interacts with the intestinal epithelium. Researchers showed that there was increased phosphorylation of vital tight (occludin and ZO-1) and adheren's (E-cadherin) junction proteins. In addition, researchers discovered that this dose of acetaldehyde in Caco-2 cells decreased protein tyrosine phosphatase (PTPase) activity (Atkinson and Rao 2001). The same group of researchers also showed that acetaldehyde (100-600 μM) increased protein tyrosine phosphatase phosphorylation of occludin, E-cadherin, ZO-1 and β-catenin in human colon samples. Therefore, acetaldehyde caused the redistribution of TJ and adheren's junction proteins, increasing intestinal epithelium permeability, due to PTPase and tyrosine phosphatase modulation (Basuroy, Sheth et al. 2005).

Excess acetaldehyde may have serious consequences in the intestine. For example, it can alter the permeability of the mucosa and thus promote the translocation of bacteria. Indeed, endotoxemia, known to be present following acute ethanol use in humans may be important in the causation of cirrhosis (Bode, Kugler et al. 1987). Therefore, an important component in the metabolism of ethanol includes the alteration of TJs caused by the primary toxic metabolite of ethanol metabolism, acetaldehyde (Ferrier, Berard et al. 2006).

### Variations in ethanol metabolism rate

There is a high variability of ethanol metabolism between individuals due to the previously mentioned physiological variables as well as by the dose of ethanol, food intake, body weight, drinking pattern, age, and sex (Stockwell and Room 2012). Men and women achieve different BAC when exposed to the same dose of alcohol per unit of body weight which is in part due to the higher body fat percentage in women, leading to higher BAC in women. There is also higher first pass metabolism in men as well (Cederbaum 2012). Ethanol metabolism also varies based on previous alcohol use in each individual. Overall, ethanol is generally metabolized at approximately one standard drink per hour (Cederbaum 2012).

## THE EFFECT OF ETHANOL ON INTESTINAL STRUCTURE AND FUNCTION

Common intestinal complications of chronic alcoholics include malnutrition and diarrhea. The malnutrition stems from dietary insufficiencies in addition to decreased nutrient absorption as discussed above. The diarrhea often stems from increased permeability of the TJs seen during chronic alcohol consumption which leads to the passive diffusion of solutes like sodium and chloride back into the lumen, causing water to follow (Ferrier, Berard et al. 2006). This diffusion of solutes, coupled with decreased activity of mucosal disaccharidases, causes the diarrhea. The following section describes the effect of ethanol on intestinal structure and function, as these changes can lead to larger symptoms as with diarrhea (Bujanda 2000).

### Effect of ethanol on intestinal function

Ingested food must pass through the GI tract which requires the muscular, endocrine, and enteric systems to produce contractions in the intestine to move the ingested material through the GI tract. Chronic alcohol consumption alters bowel motility, which could stem from ethanol-based alterations to contractile proteins, vagal function, or impairment of neuroendocrine factors

(Bujanda 2000). These results are also ileal-specific. Researchers showed that a 3 to 5% dosage of ethanol in the ileum can decrease slow wave activity, which inhibited intestinal contractions in a dose-dependent manner (Subramanya, Stephen et al. 2015).

**Effect of ethanol on the intestinal microbiota**

Since ethanol affects the motility of the GI tract, ethanol may also affect the composition of the intestinal microbiota. It has been shown that ethanol creates bacterial overgrowth in the small intestine (Hauge, Persson et al. 1997). In human subjects, almost half of chronic alcoholics had an increase in the total number of bacteria in the jejunum and duodenum of the small intestine (Hauge, Persson et al. 1997). More specific microbiota compositional changes have also been recorded. The intestinal microbiota in mice exposed to 30.9 g/kg of ethanol were investigated by 16S rRNA from cecum fecal samples. Overall, they found that ethanol increased *Bacteroidetes* and decreased *Firmicutes* presence (Yan, Fouts et al. 2011). A similar human study using mucosa sigmoid biopsies from chronic alcoholics showed an increase in *Proteobacteria* and a decrease in *Bacteriodetes*, which differed from the previous mouse study, using 16S rRNA (Mutlu, Gillevet et al. 2012). A similar human study conducted with fecal samples found the same compositional changes in the microbiota (Chen, Yang et al. 2011). Researchers even compared the composition of the microbiota when exposed to red wine consumption to gin and de-alcoholized red wine in humans (Queipo-Ortuno, Boto-Ordonez et al. 2012). Researchers found that each of these beverages changed the composition of the microbiota differently. In all, alcohol clearly causes bacterial overgrowth and compositional changes in the microbiota, which depends on many factors, even on the type of alcohol consumed (Engen, Green et al. 2015). Bacterial overgrowth and microbiota changes may

contribute to increased endotoxin translocation, causing liver damage, or even damage the mucosa, contributing to alcohol-dependent malnutrition (Bode and Bode 2003).

**Effect of ethanol on intestinal structure**

Acute doses of ethanol can damage the mucosa through a loss of epithelial cells at villus tips, reduced villus height, reduced mucosal surface area per villi, increased intra-epithelial mononuclear cells, goblet cell hyperplasia, presence of hemorrhagic erosions and hemorrhage in the lamina propria (Beck and Dinda 1981). Moreover, fluid-filled blebs at the tips of villi form when exposed to 2.1 – 4.8% ethanol in the perfused hamster jejunum as well as in human tissues which was reversible within 45 minutes (Beck and Dinda 1981). In some studies, the blebs had ruptured (Beck and Dinda 1981).

In further studies, chronic alcohol use in 120 male alcoholics consuming more than 80 g of ethanol per day were monitored for three months. Duodenal biopsies showed partial villus atrophy, lamina propria infiltrate, intraepithelial lymphocytes with wide intracellular junctions, distorted microvilli, increased endoplasmic reticulum and increased and dilated mitochondria (Bhonchal, Nain et al. 2008).

Overall, there is vast and varied structural damage in response to ethanol along the small intestine. Although some studies illustrated the damage occurs in a dose-dependent manner, the level and types of damage varied heavily between species, ethanol dosage, and intestinal segment examined. Furthermore, the exact mechanism of these changes is still well debated (Bode and Bode 2003).

**Effect of ethanol on cellular structure**

In addition to the function and structural changes in the intestine, ethanol has distinct subcellular effects as well. Perfusion of a binge-like dose of ethanol (2.1 – 4.8%) in the hamster

jejunum resulted in the swelling of the cells located at the villus tips (Beck and Dinda 1981). The swelling could be reversed in 45 minutes and the mechanism remains unclear (Beck and Dinda 1981). Electron microscopy also showed wide intracellular junctions, increased and dilated mitochondria, distorted microvilli, and increased mature epithelium at villus tips in duodenal biopsies from male chronic alcoholics (Bhonchal, Nain et al. 2008). Overall, there are also subcellular changes in response to ethanol, including those discussed below.

## The effect of ethanol on subcellular structures

### The effect of ethanol on membrane fluidity

Ethanol is amphiphilic which means that ethanol can passively diffuse through cell membranes. When this small short-chained alcohol inserts itself into the plasma membrane of cells, the hydrophobic portion of ethanol interacts with the lipid hydrocarbon chains of the plasma membrane. Meanwhile, the polar hydroxyl group of ethanol forms hydrogen bonds with the polar lipid atoms of the plasma membrane. This insertion creates a reaction across the plasma membrane, changing the fluidity of the membrane (Dickey and Faller 2007). This insertion can occur in 400 nanoseconds (Patra, Salonen et al. 2006). Researchers showed that ethanol increases membrane fluidity and decreases bilayer thickness as it traverses the membrane in computer simulations (Patra, Salonen et al. 2006, Dickey and Faller 2007). This result was shown again in *Oenoccus oeni* cells (Da Silveira, Golovina et al. 2003). Any changes in membrane fluidity, such as changing bilayer thickness, can alter the conformation of transporters in the plasma membrane (Dickey and Faller 2007). Changes in membrane fluidity can even play a role in altering Na-K-ATPase activity (Da Silveira, Golovina et al. 2003). Therefore, it is important to fully understand ethanol's effect on the plasma membrane of cells.

Ethanol also causes the membranes to become more disordered. Ethanol increases the rotation of phospholipids and the wobbling of acyl chains of phospholipids. It has been hypothesized that although significant membrane alteration occurs at 10 to 20 mM of moderate ethanol exposure, the amount of disorder caused is similar to that of a one-degree temperature change. Nevertheless, it has been suggested that the membrane order can adapt to continuous exposure to drugs like ethanol. Mice resistant to intoxication have less of an effect on membrane disorder than ethanol-sensitive mice (Goldstein 1987). Overall, more research is clearly needed to fully understand the effect of ethanol on the plasma membrane and its involvement with ethanol tolerance.

**Effect of ethanol on TJs**

Ethanol has been shown to modify TJ proteins. TJ proteins are imperative in maintaining the intestinal epithelium as a selective barrier to the lumen. TJ manipulation leads to a leaky intestinal lining, endotoxin translocation, and then liver damage commonly associated with chronic alcohol use (Ferrier, Berard et al. 2006). Intestinal epithelial permeability in response to ethanol is commonly decreased. For example, in the colon, increased intestinal permeability was seen in concentrations of ethanol above 5%, leading to a 10% decrease in colonic transepithelial resistance (Ferrier, Berard et al. 2006). Sprague Dawley rats treated with 3 g/kg ethanol, leading to BAC levels of approximately 0.08% two hours after the treatment, showed increased $^{51}$Cr-EDTA permeability, but not in doses less than 3 g/kg. Furthermore, use of antibiotics and mast cell stabilizers lessened the effect of ethanol on the permeability of the epithelial cells (Ferrier, Berard et al. 2006). Even though these studies were conducted in the colon, similar studies found ileal permeability is also similarly affected by acetaldehyde as previously discussed above (Chaudhry, Samak et al. 2015).

This increase in intestinal permeability may be due to TJ stability. Ethanol at concentrations less than 10% caused myosin light chain kinase upregulation and actin modulation by removing ZO-1 and occludin from the TJs in Caco-2 cells and the duodenal tissue of healthy humans (Elamin, Masclee et al. 2014). This process was reversible with the removal of ethanol (Elamin, Masclee et al. 2014).

Further studies conducted by the same group showed that in Caco-2 cells, a binge-like dose of ethanol exposure (40 mM) for five minutes caused a calcium-dependent activation of the Rho/ROCK pathway which leads to intestinal barrier disruption through JNK2 (Elamin, Masclee et al. 2014). The influx of calcium through L-type voltage-gated calcium channels led to the redistribution of occludin-1, E-cadherin, β-catenin and F-actin from the tight and adheren's junctions which was reversed with the Rho/ROCK inhibitor Y-27632 (Elamin, Masclee et al. 2014). Furthermore, butyrate supplementation (10 mM tributyrin) after gut injury in mice (C57BL/6J) treated with a chronic 32% ethanol-diet for 25 days showed no change in the presence of ZO-1 and occludin in the TJ (Cresci, Bush et al. 2014). The supplementation of tributyrin protected these proteins from the effect of ethanol. Overall, ethanol affects TJ proteins, which can contribute to a leaky intestinal barrier and nutrient malabsorption, in a variety of ways that can be reversed using Rho/ROCK inhibitors or tributyrin supplementation.

**Zinc and TJ regulation**

Zinc seems to play a role in the ethanol-mediated intestinal barrier disruption. Mice (C57BL/6J) were fed a Lieber DeCarli ethanol diet for four weeks and showed reduced ileal zinc concentration and increased reactive oxygen species (ROS) in the ileum, but not the jejunum or duodenum (Zhong, McClain et al. 2010). Ethanol also decreased ZO-1, occludin and claudin-1 expression in TJs. Researchers suggested that this zinc deficiency may play a role in this TJ

disassembly. More importantly, this research showed that the ileum, not the upper small intestine, plays a greater role in endotoxemia development (Zhong, McClain et al. 2010). Further studies by the same group showed that chronic ethanol, administered in the same way, reduced hepatocyte nuclear factor-4α (HNF-4α) expression, mostly in the ileum (Zhong, McClain et al. 2010). When HNF-4α was knocked down in Caco-2 cells, TJ mRNA and protein expression levels decreased, and intestinal permeability increased, as measured by transepithelial resistance. This study suggests that decreased nutrient absorption of zinc in response to ethanol reduced HNF-4α expression and then leads to TJ dysregulation and intestinal permeability (Zhong, Zhao et al. 2010). Furthermore, supplementation of zinc in an alveolar epithelial monolayer from Sprague Dawley rats fed a Lieber DeCarli diet for six weeks displayed increased epithelial barrier function, TJ protein expression and localization (Joshi, Mehta et al. 2009). Altogether, these reports show that decreased TJ protein expression following ethanol exposure can be reversed with correct nutritional absorption of zinc.

**Effect of ethanol on cellular stress and immune responses**

Since ethanol creates cellular and structural damage along the small intestine, it is reasonable to assume that an immune response is initiated in response to ethanol. In fact, leukocyte infiltration is present in jejunal segments perfused with a binge-like dose of 6% ethanol (Dinda, Kossev et al. 1996). As a secondary cascade, histamine is released from mast cells. This process quickly reverses itself within days of abstinence in chronic alcoholics. Reversal of increased B-lymphocytes and mononuclear macrophages in the lamina propria was also present within 5-10 days of abstinence in human chronic alcoholics (Bode and Bode 2003).

Ethanol has been shown to induce stress-related proteins as well as modulating the immune response. The increased expression of heat shock protein (HSP) 70 was shown in a

dose-dependent manner in mice given a 2.75, 5.5, and 8.25 g/kg ethanol for one hour (Lee, Choi et al. 2014). HSP40, HSP32, and HSP90 levels were also altered, but the changes depended on the intestinal segment and ethanol concentration. In the same study, interleukin-1β, tumor necrosis factor-α (TNF-α), and cyclooxygenase-2 (COX-2) were all increased in response to all the ethanol doses. In all, ethanol is causing stress and immune responses to occur in intestinal epithelial cells, due to the presence of stress-related HSPs and immune system modulators like TNF-α (Lee, Choi et al. 2014).

These studies are only a small picture of the immune and stress responses engaged by ethanol. There are a large amount of ethanol studies detailing the different immune and stress responses (Bode and Bode 2003). It is important to consider these responses in future ethanol studies.

**Ethanol and reactive oxygen species (ROS) production**

ROS are produced from ethanol metabolism. One pathway for ROS generation is during the production of hydrogen peroxide, in which oxygen can react with iron or copper ions to form hydroxyl radicals. Ethanol also alters complex I and complex III of mitochondria, which can lead to ROS production (Ishii, Kurose et al. 1997). Alterations in $NAD^+$/NADH ratio may exaggerate mitochondrial superoxide generation by increasing electron flow along the electron transport chain (Ishii, Kurose et al. 1997). ROS can damage many cellular functions and structures, including those at the cell membrane. Since ethanol has already been shown to alter TJ permeability, the production of ROS in response to ethanol should be noted.

Ethanol increased lipid peroxidation and reduced the antioxidant enzyme glutathione peroxidase in male Marchigian Sardinian alcohol-preferring rats administered 10% ethanol for 10 weeks (Gabbianelli, Cifani et al. 2007). No change in superoxide dismutase was recorded

(Gabbianelli, Cifani et al. 2007). In another study, rabbits were given a heavy dose of 5 g/kg ethanol for six weeks and lipid peroxidation production was measured with TBARS (Rasic-Markovic, Krstic et al. 2008). Lipid peroxidation increased in the ethanol-treated rabbit erythrocytes (Rasic-Markovic, Krstic et al. 2008). Focusing in on the GI tract, in Caco-2 cells exposed to 0-15% ethanol, an increase in oxidative stress and inducible nitric oxide synthase (iNOS) occurred as well (Banan, Choudhary et al. 1999). Similarly, researchers found an increase in iNOS activity in the ileum and colon in rats administered a heavy dose of 20% ethanol for eight weeks (Krecsmarik, Izbeki et al. 2006). A decrease in constitutive nitric oxide synthase (cNOS) was also present which is important because nitric oxide inhibits the enteric nervous system which can lead to the motility disorders associated with chronic alcohol use (Krecsmarik, Izbeki et al. 2006).

## CONCLUSION

Alcohol is commonly used by the majority of the population of the United States. However, it's use leads to negative health consequences including alcohol-dependent malnutrition. Ethanol does decrease the absorption of many nutrients including thiamine, folate, riboflavin, vitamin C, glucose and more in the small intestine. However, the mechanism of action of ethanol on decreased nutrient transport is unclear which may be in part due to the primary toxic metabolite of ethanol, acetaldehyde, or due to the changes the small intestine experiences in response to ethanol including changes to the structure and function of the small intestine, changes to the microbiota composition, increases in ROS generation and alterations to plasma membrane fluidity, TJs, and epithelial permeability. Clearly, alcohol consumption leads to complicated, direct and indirect actions on many bodily processes (Figure 3). More research is necessary to fully understand ethanol's effect on nutrient absorption at the small intestine.

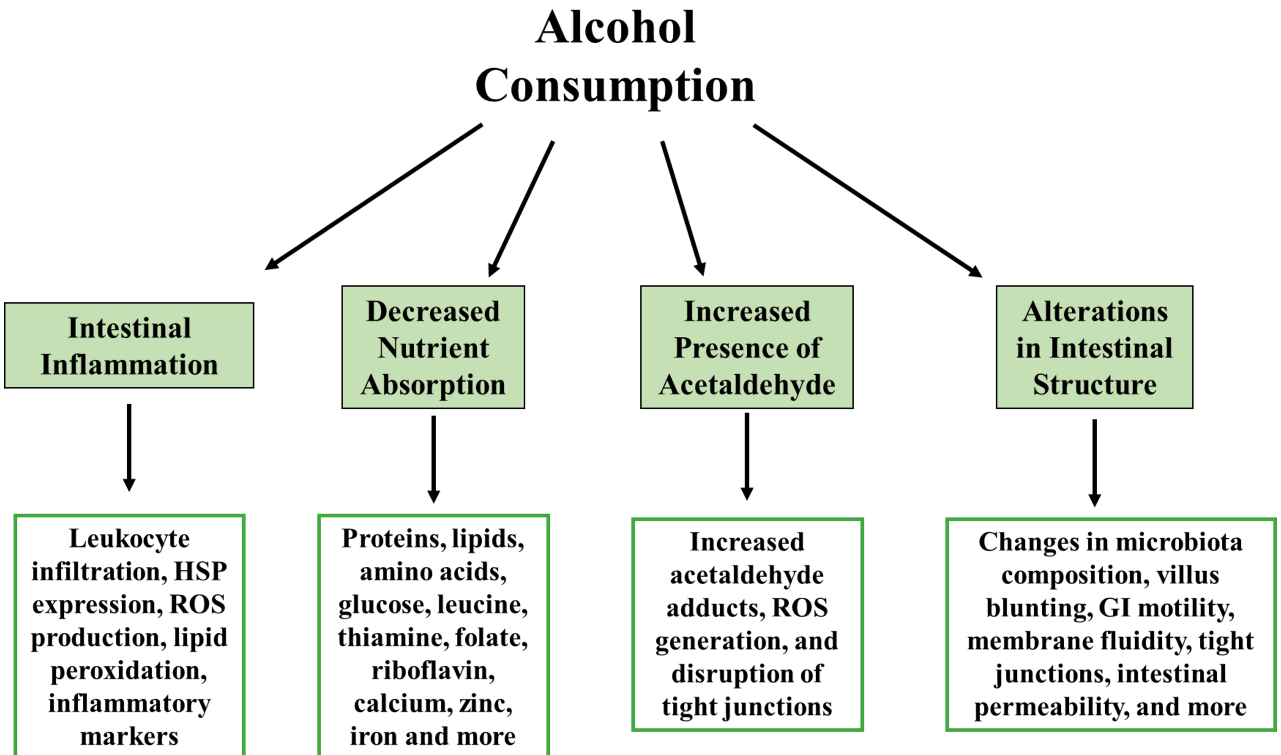


**Figure 3: The effects of alcohol consumption along the small intestine.** Alcohol consumption can lead to a wide variety of negative cellular responses including intestinal inflammation, decreased nutrient absorption, increased acetaldehyde production and alterations in intestinal structure and function. ROS – reactive oxygen species, HSP – heat shock protein, GI – gastrointestinal tract.

# CHAPTER 2

# GLUTAMINE AND GLUCOSE ABSORPTION ALONG THE SMALL INTESTINE

## Glutamine

Glutamine is a conditionally essential amino acid, meaning that glutamine can be synthesized adequately by the body, but in certain tissues is required for optimal health, especially in the intestine (Lacey and Wilmore 1990). However, this definition does not provide adequate emphasis to express glutamine's importance in the mammalian body. Glutamine helps maintain normal function of the intestine, kidney, liver, heart, brain, pancreas, and immune system. Glutamine is involved in insulin secretion, cytokine production, apoptosis, redox potential, cell stress responses, protein degradation and synthesis, extracellular matrix formation, cellular proliferation, and cellular metabolism. Furthermore, glutamine is used as a substrate and regulator in gluconeogenesis, the TCA cycle, the urea cycle, and lipogenesis. Glutamine is also a precursor for nucleotides. Clearly, this amino acid has wide ranging roles across the mammalian body (Curi, Lagranha et al. 2005).

### Glutamine's role along the intestine

In addition to being involved in many important bodily functions, glutamine is the primary fuel source for the small intestine. The intestinal epithelium must constantly renew itself, and these rapidly dividing cells use glutamine as a reliable source of energy production. Overall, approximately 70% of consumed glutamine is absorbed and catabolized by enterocytes (Pochini, Scalise et al. 2014). In addition to its roles in intestinal cell proliferation, glutamine also protects the intestinal epithelium from ROS. Glutamine is a precursor for the antioxidant glutathione, which helps prevent ROS damage. Glutamine also activates signaling mediators that regulate cellular differentiation and mucin production, which protects the integrity of the

intestinal epithelium (Kim 2011). Glutamine can induce the expression of the cellular stress response proteins like HSPs and decreases the immune response, through decreasing toll-like receptor 4 induction (Kim 2011). These processes help regulate the stress and immune responses in the intestinal barrier. Due to this wide range of protective functions in the intestine, glutamine is an important nutrient to fully investigate (Kim 2011).

**B0AT1**

Glutamine is absorbed along the small intestine through the sodium-dependent glutamine co-transporter B0AT1. B0AT1 (*SLC6A19*) belongs to the B0 family, which has a broad specificity to neutral amino acids. Several other transporter families are also selective for glutamine, including the SLC1, 6, 7, and 38 (Pochini, Scalise et al. 2014). B0AT1 also transports leucine, valine, methionine, phenylalanine, tryptophan, threonine and histidine (Broer 2009).

B0AT1 is an electrogenic co-transporter that is pH-sensitive. B0AT1 transports sodium and glutamine at a 1:1 ratio but cannot transport lithium. This co-transporter functions based on sodium. Sodium travels along its concentration gradient into the cell while glutamine is transported against its concentration gradient into the cell through B0AT1. This sodium gradient is set by the Na-K-ATPase on the BLM of enterocytes. Therefore, B0AT1 functions from secondary active transport (Pochini, Scalise et al. 2014).

B0AT1 has 12 transmembrane domains. Uncharged amino acids are favored in the hydrophobic pocket of B0AT1 formed by a hypothesized leuT fold. There are five predicted N-glycosylation sites, an intracellular phosphorylation site and two possible metal binding motifs in the middle of the co-transporter (Pochini, Scalise et al. 2014). Nimesulide and benztropine are two competitive inhibitors of B0AT1 (Cheng, Shah et al. 2017).

**B0AT1 localization**

B0AT1 is localized in the proximal tubules, kidney, skin, pancreas, prostate, stomach, liver and the BBM of intestinal microvilli. Specifically in the small intestine, this co-transporter was localized via immunohistochemistry to the villus cells but not to the secretory crypt cells of the rabbit small intestine (Talukder, Kekuda et al. 2008).

**B0AT1 and disease**

Mutations in B0AT1 lead to an autosomal recessive disorder called Hartnup disorder. Occurring more frequently in European populations, Hartnup disorder is often asymptomatic. However, clinical aspects include renal aminoaciduria presenting with varying symptoms, including a distinctive skin rash which is caused by the malabsorption of neutral amino acids, absorbed by co-transporters in the B0 system, in the kidney (Broer 2009).

In the small intestine, B0AT1 has also been shown to be affected through the induction of chronic intestinal inflammation (Singh, Arthur et al. 2015, Arthur, Singh et al. 2018, Singh, Arthur et al. 2018). Glutamine absorption was reduced in response to conditions of chronic intestinal inflammation in rabbits through a reduction in co-transporter number. This alteration was reversed with the application of glucocorticoids, a broad spectrum immune modulator (Arthur, Saha et al. 2012) and a mast cell stabilizer ketotifen (Singh, Arthur et al. 2015). The cyclooxygenase pathway was determined to be the primary regulator of this phenomenon (Arthur, Singh et al. 2018).

**B0AT1 and ethanol**

There is no current literature on the effect of ethanol on the sodium-dependent glutamine co-transporter B0AT1.

**Glucose**

Glucose is a monosaccharide and is the primary fuel source for mammalian metabolism. Glucose travels down the GI tract often as a complex carbohydrate. These complex carbohydrates are broken down by amylases secreted mainly by the pancreas. These complex carbohydrates are broken down into monosaccharides like glucose and are then absorbed into enterocytes (Lehmann and Hornby 2016). Glucose is then often stored as glycogen or travels in the vascular system to be used as necessary.

Glucose is used as a fuel source in multiple metabolism processes including glycolysis, the pentose phosphate pathway, the citric acid cycle (Krebs cycle), aerobic respiration and anaerobic respiration. The breakdown of glucose through these cycles leads to varying amounts of energy in the form of ATP. Furthermore, glucose can be broken down and built into required molecules such as lipids, vitamin C and glycogen.

**SGLT1**

The primary route of glucose absorption is through a sodium-dependent glucose co-transporter located at the BBM of enterocytes called SGLT1 (*SLC5A1*). SGLT1 is part of the SLC5 family, which transports sodium with glucose and galactose (Vrhovac, Balen Eror et al. 2015). In fact, SGLT1 was first cloned in 1987 (Hediger, Coady et al. 1987) and was the first member of the SLC5 family. Transporters in this family also move glucose, fructose, inositol, short-chain fatty acids, iodide, choline and biotin (Wright, Ghezzi et al. 2017).

Researchers showed that SGLT1 is the primary glucose co-transporter in the small intestine using BBMVs from wild-type and knock out SGLT1 mice. Compared to wild-type mice, there was no detectible uptake of the specific glucose co-transporter substrate α-methyl-*D*-

glucoside in the knock out mice, showing that SGLT1 is the predominate form of glucose co-transport in the small intestine (Gorboulev, Schurmann et al. 2012).

SGLT1 is composed of 664 amino acids and has 14 transmembrane domains. SGLT1 transports at a 2:1 ratio of sodium to glucose which means that SGLT1 has two sodium binding sites (Hediger, Coady et al. 1987). When sodium and glucose bind, this closes external gates and allows the protein to isomerize to move the molecules inward. When the molecules of sodium and glucose release, the protein reverts externally, which takes in total approximately 20 milliseconds. It is hypothesized that transmembrane domains one, two and ten compose the outer gates made of hydrophobic side-chains (Wright, Ghezzi et al. 2017).

SGLT1 is considered sodium-dependent, which means that it transports sodium along its concentration gradient into the cell, which allows the co-transporter to move glucose against its concentration gradient into the cell. Since SGLT1 is a sodium-dependent co-transporter, it relies on the BLM-located Na-K-ATPase to maintain the sodium gradient; therefore, this process occurs through secondary active transport. Furthermore, SGLT1 is coupled with the main Na/H exchanger 3 (NHE3) in the small intestine (Coon, Kekuda et al. 2008, Lehmann and Hornby 2016).

This electrogenic co-transporter also draws water into the cell. Researchers estimate that approximately 70% of the passive water flow into the cell is based off the action of SGLT1. It has been suggested that water co-transports with glucose and sodium, but this point is still contested today (Wright, Ghezzi et al. 2017).

### SGLT1 inhibition

Due to glucose's role in type II diabetes and obesity, SGLT1 inhibitors have been well studied. Phlorizin is one of many SGLT1 inhibitors. Phlorizin binds to the external surface of SGLT1, only when sodium is bound (Wright, Ghezzi et al. 2017).

### SGLT1 localization

SGLT1 is expressed throughout the small intestine, with little to no SGLT1 expression present in the rest of the GI tract. Lower levels are present in the ileum than the rest of the small intestine (Lehmann and Hornby 2016). mRNA expression of SGLT1 is present in the intestine, kidney, brain, spinal cord, spleen, lung, liver, uterus, and pancreas. SGLT1 is expressed in the human small intestine, liver, lung and heart (Vrhovac, Balen Eror et al. 2015).

### SGLT1 and disease

Overall, SGLT1 has a wide applicability in a host of diseases including cholera, type II diabetes and obesity. During conditions of type 2 diabetes, SGLT1 protein expression levels are increased (Lehmann and Hornby 2016). SGLT1 may have regulatory roles in conditions of obesity, as knocking down the SGLT1-regulatory protein RS1 led to an increase in expression of SGLT1 and corresponding glucose uptake (Lehmann and Hornby 2016). On the other hand, lacking SGLT1 presents in the rare glucose-galactose malabsorption characterized by severe and life-threatening diarrhea (Lehmann and Hornby 2016). At the small intestine, recent studies have demonstrated that chronic intestinal inflammation decreases sodium-dependent glucose co-transport, but not Na/H exchange in rabbit intestinal villus cells which is due to a decrease in the number of BBM-located SGLT1 co-transporters (Manoharan, Sundaram et al. 2018). Furthermore, exposure to peroxynitrite also inhibited expression of SGLT1 without changing the

expression of NHE3, due to a p38 mitogen-activated protein kinase pathway (Manoharan, Sundaram et al. 2018). Overall, SGLT1 is involved in a variety of different disease states.

**The effect of ethanol on SGLT1**

The effect of ethanol on glucose absorption was previously detailed in chapter one. Overall, researchers showed that ethanol decreases glucose absorption in the small intestine. However, no studies have been conducted directly on SGLT1 using a moderate dose of ethanol.

**Ethanol's effect on the Na-K-ATPase**

As absorption of glutamine and glucose along the small intestine are sodium-dependent processes, it is important to consider the effect of ethanol on the Na-K-ATPase. The Na-K-ATPase moves three sodium ions out of the cell and two potassium ions into the cell with energy from ATP. The Na-K-ATPase is located on the BLM of intestinal epithelial cells which establishes the low concentration of sodium in enterocytes that the sodium-dependent nutrient co-transporters rely on (Bode and Bode 2003). Therefore, it is important to consider the effect of ethanol on Na-K-ATPase activity. Studies on Na-K-ATPase activity in response to ethanol have varied based on the tissue and dosages used. In renal cells, 150 mM ethanol showed decreased Na-K-ATPase activity in response to acute ethanol and increased activity of the Na-K-ATPase at chronic doses. A further study by the same group found that chronic ethanol consumption (20% ethanol for 10 weeks) in renal cells from rats rose due an increase in catalytic units (Rodrigo, Novoa et al. 1996) and an increase in $V_{max}$ parameters which suggests that chronic ethanol stimulated Na-K-ATPase activity through an increase in the number of catalytic subunits, specifically the α1 subunit (Rodrigo and Thielemann 1997). The increased activity of the Na-K-ATPase also varied based on the region of the kidney (Rodrigo, Vergara et al. 1991). In another study, rabbits given 5 g/kg ethanol for six weeks showed that chronic ethanol consumption

increased Na-K-ATPase activity in erythrocytes (Rasic-Markovic, Krstic et al. 2008). This increase in Na-K-ATPase activity in response to chronic alcohol was shown again in rat skeletal muscle, erythrocytes (Johnson and Crider 1989), lung (Otis, Mitchell et al. 2008), liver (Pascale, Daino et al. 1989) and in human chronic alcoholics' erythrocytes (Johnson and Crider 1989). Researchers hypothesized that this may be due to the changes in membrane fluidity seen in response to ethanol (Johnson and Crider 1989). The effect of ethanol on the Na-K-ATPase activity in the intestine was previously described in the jejunum of hamsters, where no chronic change in Na-K-ATPase activity was determined, but an acute dosage inhibited the activity of the Na-K-ATPase (Hoyumpa, Patwardhan et al. 1981). In all, there is a wide range of research describing the effect of ethanol on the Na-K-ATPase in a variety of tissues across the body.

**Conclusions**

The two main fuel sources for the small intestine and the mammalian body, glutamine and glucose, are absorbed through sodium-dependent nutrient co-transporters: B0AT1 and SGLT1, respectively. These transport systems are reliant on the Na-K-ATPase to set the sodium gradient in intestinal epithelial cells (Figure 4). However, Na-K-ATPase activity is decreased in response to chronic ethanol in many tissues, but not in the intestine. How ethanol directly affects B0AT1 and SGLT1 is not known. Further research must be conducted on the effect of ethanol on B0AT1 and SGLT1 in the small intestine in order to better understand the onset of alcohol-dependent malnutrition.

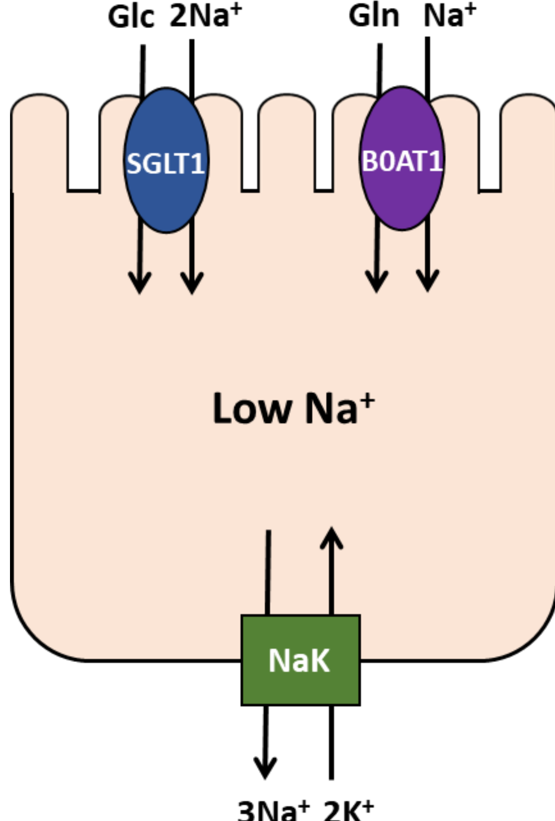


**Figure 4: The sodium-dependent glutamine and glucose co-transporters B0AT1 and SGLT1, respectively.** The sodium-dependent glucose co-transporter SGLT1 moves two sodium ions into the cell along with one glucose molecule, while B0AT1 draws one sodium ion into the cell, along its concentration gradient, and one glutamine molecule against its concentration gradient. The Na-K-ATPase sets the concentration gradient on the BLM by exchanging 3 sodium ions out of the cell and 2 potassium ions into the cell. NaK – Na-K-ATPase, $Na^+$ - sodium, $K^+$ – potassium, Glc – glucose, Gln - glutamine.

# CHAPTER 3

## ESTABLISHING AN ETHANOL DOSE

The initial steps of this dissertation required establishing the best dosage of ethanol. A range of ethanol dosages, between a 0.02% and a 0.08% BAC were tested, as few studies have examined the effect of ethanol at or below the legal limit in the United States. We determined that one and three hours of the equivalent of a 0.02% BAC dosage of ethanol significantly decreased glutamine uptake in IEC-18 cells (Figure 5; 1265±55.9 pmol/mg protein•min in controls vs 793±70.0 ethanol-treated cells at 1-hour, $p<0.01$, n=4). The methods for this procedure are described in chapter 4.

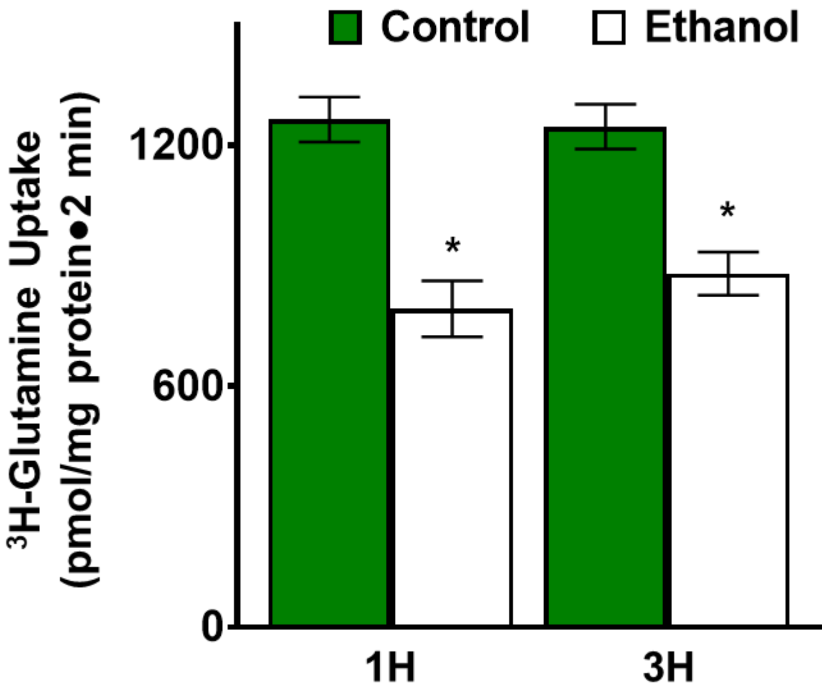


**Figure 5: A dosage of ethanol equivalent to a 0.02% BAC significantly decreased sodium-dependent glutamine uptake in IEC-18 cells after one and three hours (*, $p<0.01$, n=4).** Error bars represent the standard error of the mean (SEM). The p-values were adjusted for multiple hypotheses using a Bonferroni correction (1H: $p<0.01$; 3H: $p<0.01$).

Furthermore, a dosage of ethanol equivalent to a 0.04% BAC significantly decreased glutamine uptake (Figure 9; 1285±37 pmol/mg protein•2 min in controls vs 874±75 in ethanol-treated cells at 1-hour, $p<0.01$, n=6). A dosage equivalent to a 0.08% BAC significantly decreased glutamine uptake in IEC-18 cells as well (Figure 6; 1213±52.7 pmol/mg protein•min in controls vs 778.5±67.8 in ethanol-treated cells at 1-hour, $p<0.05$, n=4).

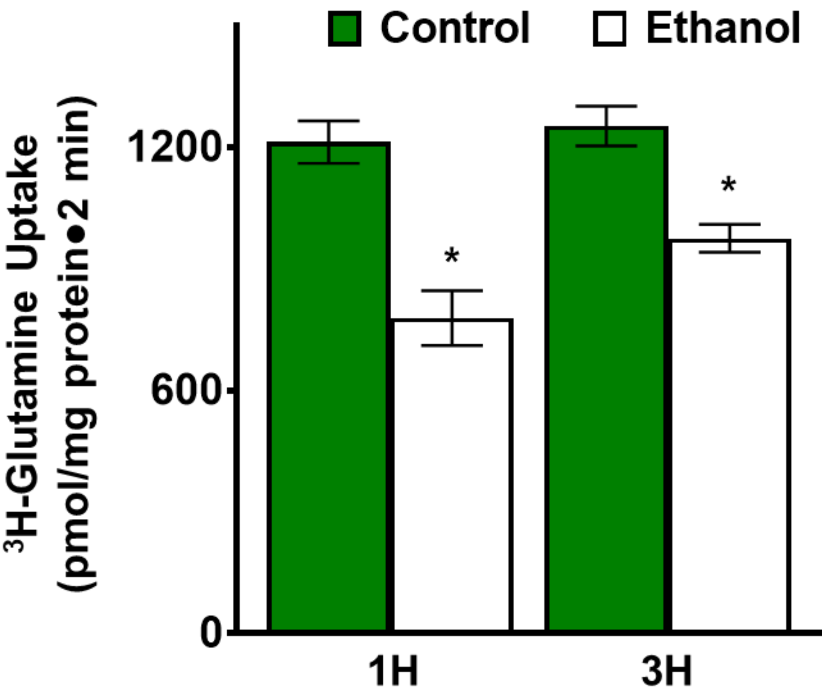


**Figure 6: A dosage of ethanol equivalent to a 0.08% BAC significantly decreased glutamine uptake in IEC-18 cells at one and three hours** (*, $p<0.01$, n=4). Error bars represent the SEM. The p-values were adjusted for multiple hypotheses using a Bonferroni correction (1H: $p=0.02$; 3H: $p<0.01$).

Initially, it was important to investigate differing concentrations of ethanol on glutamine uptake in IEC-18 cells to establish the proper dosage of ethanol and to ensure that this effect was not concentration dependent. We determined that at one hour and three hours, 0.02%, 0.04% and 0.08% ethanol all had a significant inhibition on glutamine uptake in IEC-18 cells. A dosage

equivalent to a 0.04% BAC was selected due to its translational applicability, as it is considered a moderate dose of ethanol.

## VALIDATION OF MODEL

Next, we established that this dosage of ethanol did not cause changes in cell viability using LDH and trypan blue assays (Figure 14). We also ensured that ethanol itself was causing this decrease in glutamine absorption, and not acetaldehyde, the primary toxic metabolite of ethanol metabolism (Figure 10).

One question was left in order to fully validate this model: should the ethanol treatments be on the apical or BLM? Ethanol is absorbed quickly along the duodenum, and the concentration of ethanol used in these studies would not transit the full length of the small intestine before being completely absorbed into the blood stream (Cederbaum 2012). Thus, ethanol interacts with the ileum of the small intestine along the BLM of intestinal epithelial cells (Bode and Bode 2003, Krenz and Korthuis 2012). Therefore, glutamine uptake studies were conducted in transwells, using the same methodology as described previously, in order to see if the effect of ethanol on the BLM differed from apical treatment of ethanol. The only difference in the methodology used is that ethanol was solely administered on the BLM of IEC-18 cells using transwells. Moderate ethanol treatment on the BLM of IEC-18 cells for one hour decreased glutamine uptake (Figure 7; 1313±48.8 pmol/mg protein•min in controls vs 986.7±54.5 in ethanol-treated cells at 1-hour, $p<0.05$, $n=3$).

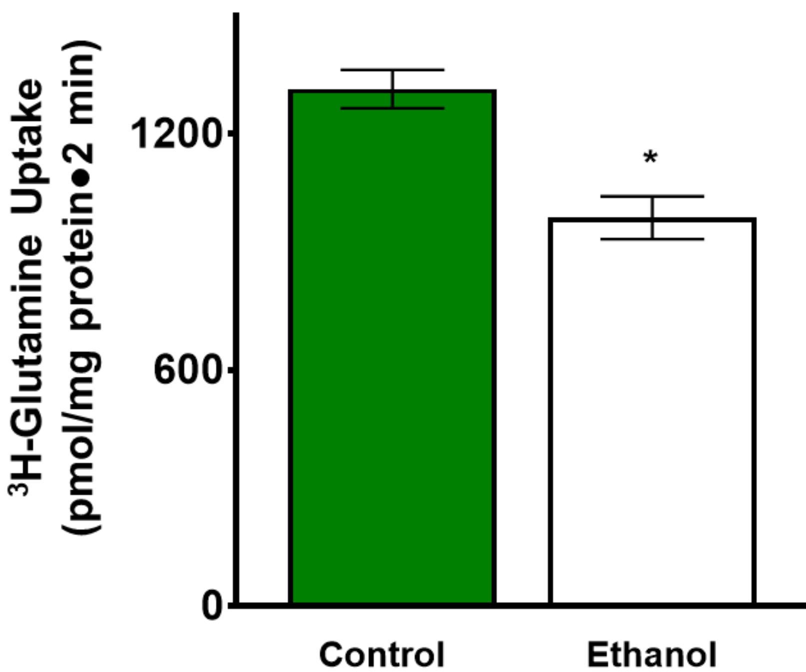


**Figure 7: Moderate ethanol treatment on the BLM decreased glutamine uptake in IEC-18 cells** (*, $p<0.05$, n=3). Error bars represent the SEM.

The percentage decrease between control and ethanol-treated cells on the luminal, or apical, side (68.0% inhibition) did not significantly differ from cells treated on the BLM (75% inhibition). Therefore, studies were conducted throughout this dissertation on the apical side, to avoid the temporal and fiscal costs associated with transwell studies. At this point, we were able to conduct experiments to elucidate the effect of moderate ethanol on glutamine and glucose absorption in IEC-18 cells.

# CHAPTER 4

## MODERATE ALCOHOL CONSUMPTION UNIQUELY REGULATES SODIUM-DEPENDENT GLUTAMINE CO-TRANSPORT IN RAT INTESTINAL EPITHELIAL CELLS

Molly Butts, Soudamani Singh, Jennifer Haynes, Subha Arthur and Uma Sundaram•

Department of Clinical and Translational Sciences and Appalachian Clinical and Translational Science Institute, Joan C. Edwards School of Medicine, Marshall University, 1600 Medical Center Drive. Huntington, WV 25701, USA

•Address Correspondence to:

Uma Sundaram MD

Joan C Edwards School of Medicine,

Marshall University

1600 Medical Center Drive

Huntington, WV 25701

Phone: 304 691 1841

Email: sundaramu@marshall.edu

Running Head: Ethanol regulates sodium-dependent glutamine co-transport

**Abstract**

Malnutrition is common in chronic alcoholics. Glutamine is a conditionally essential amino acid which is vital to maintain the health and integrity of the mammalian small intestinal mucosa. Glutamine is absorbed via Na-dependent glutamine co-transport B0AT1 (SLC6A19) located on the brush border membrane (BBM) of intestinal absorptive villus cells. How ethanol may affect B0AT1 is unknown. Intestinal epithelial cells grown to confluency were exposed to the equivalent of a 0.04% blood alcohol content (8.64 mM ethanol). Na-dependent $^{3}$H-glutamine uptakes were done to determine B0AT1 activity. Na-K-ATPase activity was measured as $P_i$ release. Western blot and immunocytochemistry (ICC) analysis were performed with the appropriate B0AT1 antibodies. Results: Ethanol significantly decreased B0AT1 activity at all time points. The Na-K-ATPase provides the favorable Na-gradient for B0AT1 and its activity was reduced by moderate ethanol at all time points. Kinetic studies showed that the mechanism of inhibition of B0AT1 by ethanol was secondary to a decrease in the maximal rate of uptake ($V_{max}$) of glutamine without a change in the affinity ($1/K_m$) of B0AT1. Western blot studies demonstrated a significant decrease in B0AT1 protein expression in the whole cell homogenate and in the BBM. ICC studies showed a similar decrease in immunofluorescence intensity. This decrease in glutamine absorption was likely mediated by protein kinase C (PKC). Conclusions: Ethanol directly inhibits the absorption of glutamine in intestinal epithelial cells by decreasing B0AT1 co-transporter numbers as well as by altering the Na-gradient possibly via PKC. Thus, these studies indicate that malnutrition associated with alcohol consumption may be at the level of the nutrient absorptive pathways of intestinal epithelial cells.

## Introduction

According to the National Institute on Alcohol Abuse and Alcoholism, over 15 million adults in the United States have an alcohol use disorder. Chronic alcohol use, defined by binge drinking five or more days per month, has many well documented negative consequences including an increased risk for cancer, cardiovascular disease, liver disease and malnutrition (Rehm, Baliunas et al. 2010, Organization 2018). This increased risk of malnutrition was demonstrated in 1984, when studies conducted by the Veterans Health Administration Cooperative Studies Program presented that patients with almost 50 percent of total energy intake from alcohol were often malnourished despite adequate caloric intake (McClain, Barve et al. 2011).

For some time, chronic alcoholics were considered to be malnourished due to suboptimal dietary intake. However, researchers have determined that ethanol decreases the absorption of essential vitamins along the small intestine (Bode and Bode 2003). The absorption of over 20 nutrients have been shown to be affected by chronic alcohol consumption which includes proteins, carbohydrates, including glucose (Chang, Lewis et al. 1967, Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981), lipids (Pfeiffer, Schmidt et al. 1992) as well as vitamins such as vitamin $B_{12}$ (Bode and Bode 2003), and vitamin C (Guo, Wang et al. 2013). Clearly, the effect of ethanol on nutrient absorption in chronic alcoholics has been well characterized. However, few studies have examined the effect of ethanol on the individual nutrient co-transporters that are responsible for absorbing these essential nutrients. Ethanol has been shown to affect vitamin co-transporters, including the thiamine (Subramanya, Subramanian et al. 2010), folate (Romanoff, Ross et al. 2007), riboflavin (Subramanian, Subramanya et al. 2013), and vitamin C co-transporters which leaves a substantial gap in knowledge surrounding

ethanol's effect on many other nutrient co-transporters along the small intestine (Guo, Wang et al. 2013).

Furthermore, in over fifty years of alcohol and nutrition research, the effect of ethanol on glutamine absorption in the small intestine has never been investigated. Glutamine is a conditionally essential amino acid that is imperative for maintaining the health of the intestinal mucosa (Pochini, Scalise et al. 2014). Glutamine is the primary source of energy for enterocytes and has been shown to play an important role in maintaining the integrity of the mucosa during gastrointestinal disorders like inflammatory bowel disease (Singh, Arthur et al. 2015, Singh, Arthur et al. 2018). It was previously shown that the primary sodium-dependent glutamine co-transporter in the BBM of villus cells is B0AT1 (*SLC6A19*) (Talukder, Kekuda et al. 2008). B0AT1 is an electrogenic transmembrane protein that functions as a secondary active co-transporter. Thus, B0AT1 moves sodium out of the cell and glutamine into the cell at a 1:1 ratio along sodium's concentration gradient (Pochini, Scalise et al. 2014). The concentration gradient for B0AT1 is provided by the basolateral Na-K-ATPase. Once in the body, glutamine is used in a variety of cell metabolism processes including as a substrate in the TCA cycle, gluconeogenesis, lipogenesis and in the urea cycle (Curi, Lagranha et al. 2005).

In this study, we hypothesized that ethanol affects the absorption of glutamine via B0AT1 in the BBM of villus cells. To better understand the onset of alcohol-dependent malnutrition, we focused our study on a moderate dose of ethanol, defined as the amount in two alcoholic beverages per day for men and one for women, equivalent to a blood alcohol content (BAC) of 0.04%. Thus, the aim of the present study is to investigate the effect of a moderate dose of ethanol on B0AT1 in rat intestinal epithelial cells.

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## Methods

### Cell culture

The immortalized non-malignant rat intestinal epithelial cell line IEC-18 (CRL-1589 American Type Culture Collection) was treated with 8.64 mM ethanol, the equivalent of a 0.04% BAC dosage, directly into fresh medium for 1, 3, 6, or 12 hours. Control-treated cells were exposed to the same volume of sterile water in fresh medium. Cells were cultured in Dulbecco's modified Eagle Medium (DMEM) (high glucose 4.5 g/l, sodium bicarbonate 3.7 g/l) containing 2 mM L-glutamine, 10% vol/vol bovine fetal serum, 0.02% insulin, and 0.25%-hydroxybutyric acid in a humidified atmosphere of 10% $CO_2$ at 37°C. Cells were grown to 4 days post confluence as monolayers, only using cells between passages 5 and 20, until ethanol treatment. Four days post confluent cells were used because the cells have the characteristics of mature villus enterocytes at this point in their development. The medium was changed every 2 to 3 days.

### Na-K-ATPase measurement

IEC-18 cells were grown and treated with ethanol as described above. Control and ethanol-treated cells were washed twice with ice-cold PBS and cells were harvested by scraping. The cells were sonicated and centrifuged at 8000 x *g* for 5 minutes. Inorganic phosphate ($P_i$) formation in cellular homogenates was measured as indirect Na-K-ATPase activity as previously described (Forbush 1983) and the activity was expressed as nanomoles of $P_i$ released per milligram protein per minute.

### Uptake studies in IEC-18 cells

As previously described (Arthur, Coon et al. 2014), sodium-dependent uptakes were conducted using Na-HEPES buffer (47 mM KCl, 1 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 20 mM HEPES, 125 mM $CaCl_2$, and 130 mM NaCl; pH 7.4). Uptakes were performed with and without

sodium using Na-HEPES and a buffer containing 130 mM TMA-Cl and Na-free HEPES. By including the sodium-free condition, the passive diffusion of glutamine can be measured. Both reaction buffers contained 200 µM of cold L-glutamine and 10 µCi $^{3}$H-*L*-glutamine. All reactions were performed for exactly 2 minutes and stopped with ice-cold TMA-HEPES. The cells were then placed in 5 mL scintillation fluid and kept overnight. Samples were measured in a scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA).

**Protein quantification**

Using the Bio-Rad DC protein assay kit (Hercules, CA), total protein was measured by the Bradford method. Bovine serum albumin (BSA) was used as a standard.

**Kinetics**

Na-dependent glutamine uptakes were performed at 30 seconds at varying concentrations of glutamine (0.1, 0.5, 1, 5, 10, 25, 75 and 100 mM) as described above. Uptake values were evaluated by Michaelis-Menten kinetics using a non-linear regression data analysis using Prism 7 software (GraphPad, San Diego, CA).

**Western blot studies**

Cells for whole cell homogenates were treated, collected and centrifuged at 8000 x *g* at 4°C for 5 minutes. The resulting pellet was solubilized in RIPA buffer containing protease inhibitors (Santa Cruz, CA). The cellular extracts in RIPA were rocked for at least one hour at 4°C and then centrifuged at 8000 x *g* at 4°C for 5 minutes. The resulting supernatant was measured for protein content using a NanoDrop Spectrophotometer (Thermo Scientific). BBM protein was collected, solubilized, and measured as described previously (Talukder, Kekuda et al. 2008, Arthur, Singh et al. 2018). Proteins were separated on a 12% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked in 5% bovine

serum albumin for 1 hour as previously described (Singh, Arthur et al. 2015, Singh, Arthur et al. 2018). Then, the membranes were incubated with rabbit polyclonal primary antibodies against B0AT1 (Abcam 180516) at 1:500 dilution at 4°C overnight. Membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies at 1:1000 dilution at room temperature for 1 hour. ECL-TM Western Blotting Detecting Reagent was used to detect the immobilized proteins. Luminescence was detected and analyzed by densitometry using a FluorChem M imager (ProteinSimple). All blots were stripped and re-probed with a mouse monoclonal primary antibody against ezrin (MilliporeSigma MAB3822-C) at 1:1000 dilution as above, and proteins were normalized against the ezrin values.

**Immunocytochemistry (ICC)**

Cells grown on glass coverslips were fixed with ice-cold methanol for 4 minutes. Non-specific binding of primary antibodies was blocked using 5% BSA and 0.1% Triton-X for 1 hour at room temperature. Cells were then washed in room temperature PBS 3 times for 5 minutes each and incubated with B0AT1 primary antibody (Abcam 180516) at 1:250 dilution for 1-hour. Cells were washed as before then incubated with goat anti-rabbit Alexa Fluor 594 secondary antibody (Invitrogen, Eugene, OR) for 1 hour. Cells were washed again, mounted with Fluoroshield Mounting Medium with DAPI (Abcam) and imaged using a 20X objective on an EVOS FL Auto 2 microscope (Invitrogen) and quantified with Alpha View version 3.4.0.0.

**siRNA transfections**

Predesigned RNA silence for a negative control (ThermoFisher AM4635) and protein kinase C (PKC) (ThermoFisher s128272 and s128273) were used for siRNA transfections. Individual siRNAs (1.5 µg each) were suspended in a nucleofector solution (pH 7.4, 7.1 mM ATP, 11.6 mM $MgCl_2.6H_2O$, 13.6 mM $NaHCO_3$, 84 mM $KH_2PO_4$ and 2.1 mM glucose). Using

IEC-18 cells, both negative controls and PKC siRNA was nucleofected into IEC-18 cells using a Nucleofector II device (Amaxa) according to the manufacturer's instructions. Cells were grown, treated and used for experiments as previously described.

**Statistical analysis**

Each number (n) for any set of experiments refers to cell preparations from different passages. Data were computed in triplicate and means ± standard error were evaluated. P-values were derived by unpaired t-tests using Prism 7 software (GraphPad, San Diego, CA). Experiments using more than one timepoint had their p-value adjusted for multiple hypotheses using a Bonferroni correction. A p-value of less than 0.05 was considered statistically significant.

## Results

### Effect of ethanol on sodium-dependent glutamine uptake in IEC-18 cells

To determine if ethanol effects glutamine absorption, $^3$H-glutamine uptake was done in IEC-18 cells. Sodium-dependent glutamine uptake was decreased in 4 days post confluent IEC-18 cells at the 1, 3, 6, and 12-hour timepoints (Figure 8; 1285±37 pmol/mg protein•2 min in controls vs 874±75 in ethanol-treated cells at 1-hour, $p<0.01$, n=6).

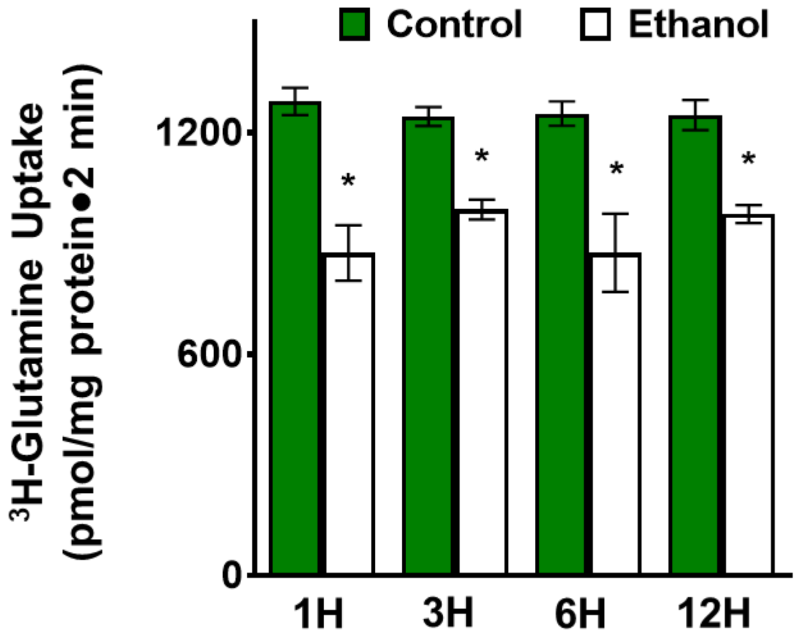


**Figure 8: Moderate ethanol decreased glutamine absorption in IEC-18 cells** (*, $p<0.05$, n=6). Error bars represent the SEM (1H: $p<0.01$; 3H: $p<0.01$; 6H: $p=0.03$; 12H: $p<0.01$).

### Effect of acetaldehyde on glutamine uptake in IEC-18 cells

The primary toxic metabolite of ethanol metabolism is acetaldehyde, which at even extremely low levels can cause cellular damage (Fisher, Swaan et al. 2010). To determine whether this decrease in glutamine absorption is due to acetaldehyde's action, 15 µM acetaldehyde was added directly to the cell culture medium of IEC-18 cells for 1-hour. A

commercial kit (Bioassays Systems) was used to determine the minimum concentration of acetaldehyde following ethanol treatment in IEC-18 cells. Glutamine uptake does not change following exposure to 15 µM acetaldehyde, suggesting that ethanol, not acetaldehyde, is the primary cause for the decrease in Na-dependent glutamine absorption (Figure 9; 1082±71.9 pmol/mg protein•2 min in control vs 1067±37.3 in ethanol-treated cells at 1-hour, $p>0.05$, n=4).

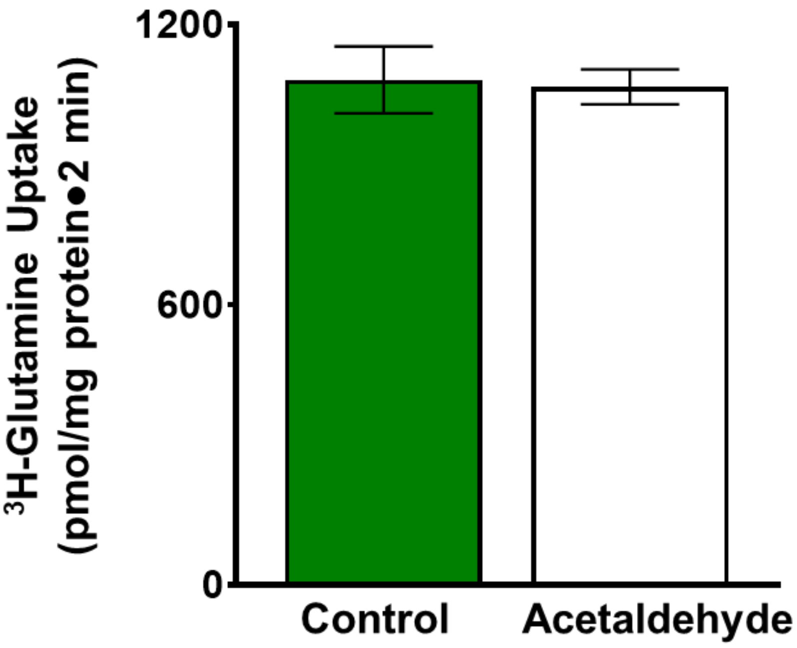


**Figure 9: Acetaldehyde did not alter $^3$H-glutamine uptake in IEC-18 cells** (n=4). Error bars represent the SEM.

**Effect of ethanol on Na-K-ATPase activity in IEC-18 cells**

The Na-K-ATPase located on the basolateral membrane (BLM) provides the transcellular sodium gradient necessary for sodium-dependent nutrient co-transporters. Na-K-ATPase activity was significantly inhibited by ethanol at all timepoints (Figure 10; 22.750±1.832 nmol/mg protein•min in controls vs 10.667±1.031 in ethanol-treated cells at 1-hour, $p<0.01$, n=6). There was no recovery in the Na-K-ATPase activity between the 1 and 12-hour time conditions.

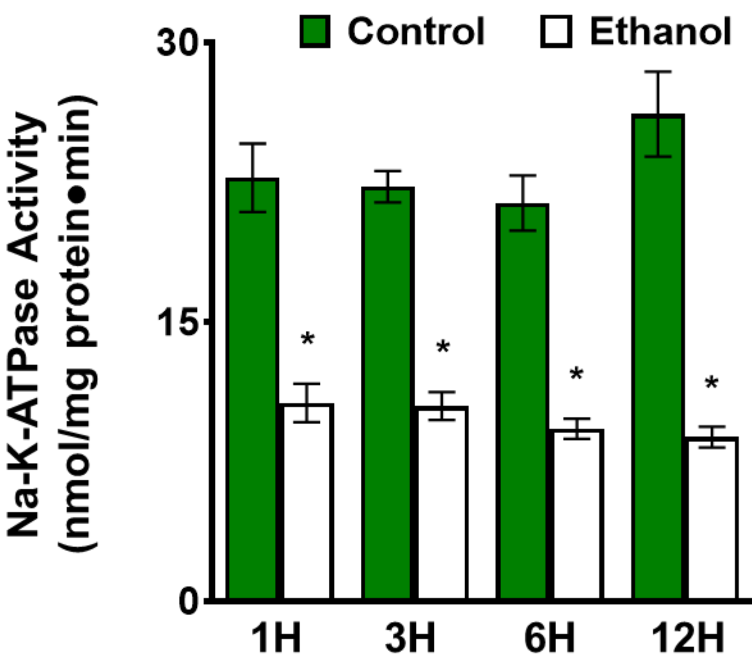


**Figure 10: Moderate ethanol decreased Na-K-ATPase activity in IEC-18 cells** (*, $p<0.05$, n=6). Error bars represent the SEM (1H: $p<0.01$; 3H: $p<0.01$; 6H: $p<0.01$; 12H: $p<0.01$).

**Effect of ethanol on the kinetic parameters of sodium-dependent glutamine co-transport in IEC-18 cells**

The effect of ethanol on Na-K-ATPase activity as well as sodium-dependent glutamine uptake was fairly linear over the 1 to 12-hour timepoints. Therefore, kinetic studies were conducted at one hour. Kinetic studies showed that the mechanism of inhibition of sodium-dependent glutamine co-transport in IEC-18 cells after 1-hour exposure to ethanol was secondary to a reduction in the maximal rate of uptake ($V_{max}$) of the co-transporter without a change in the affinity of the co-transporter ($K_m$) (Table 4). These studies indicate that the mechanism of inhibition of sodium-dependent glutamine co-transport in IEC-18 cells is likely secondary to reduced co-transporter number.

| | $V_{max}$ (nmol/mg protein•30sec) | $K_m$ (mM) |
|---|---|---|
| **Control** | 1.5±0.03 | 0.1±0.01 |
| **Ethanol** | 1.3±0.03* | 0.1±0.01 |

**Table 4: In IEC-18 cells, the maximal rate of uptake ($V_{max}$) of sodium-dependent glutamine co-transport was significantly reduced when exposed to 1-hour of ethanol** (*, p<0.01, n=4).

**Quantitation of B0AT1 protein expression in IEC-18 cells exposed to ethanol**

To determine the effect of ethanol on B0AT1 protein expression, Western blot analysis was performed on whole cell homogenates. The protein expression of B0AT1 in whole cell homogenates was significantly decreased in IEC-18 cells after 1-hour of ethanol treatment (Figure 11A) which was confirmed by densitometric quantitation (Figure 11B; ethanol-treated cells were 0.78 of controls, p<0.05, n=6).

B0AT1 is localized to the BBM of small intestinal enterocytes and therefore, Western blots using BBM were conducted. B0AT1 protein expression levels were decreased in the BBM of IEC-18 cells exposed to 1-hour of ethanol (Figure 11C), again confirmed by densitometric quantitation (Figure 11D; ethanol-treated cells were 0.81 of controls, p<0.01, n=4). These data, along with the kinetic analysis above, demonstrated that the mechanism of ethanol-mediated sodium-dependent glutamine co-transport inhibition is secondary to a decrease in B0AT1 protein expression in IEC-18 cells in the BBM.

In addition, ICC studies were performed in control and ethanol-treated IEC-18 cells exposed to 1-hour of ethanol. Consistent with the Western blot data above, B0AT1 immunofluorescence intensity was significantly decreased in cells exposed to ethanol (Figure 12A, 12B).

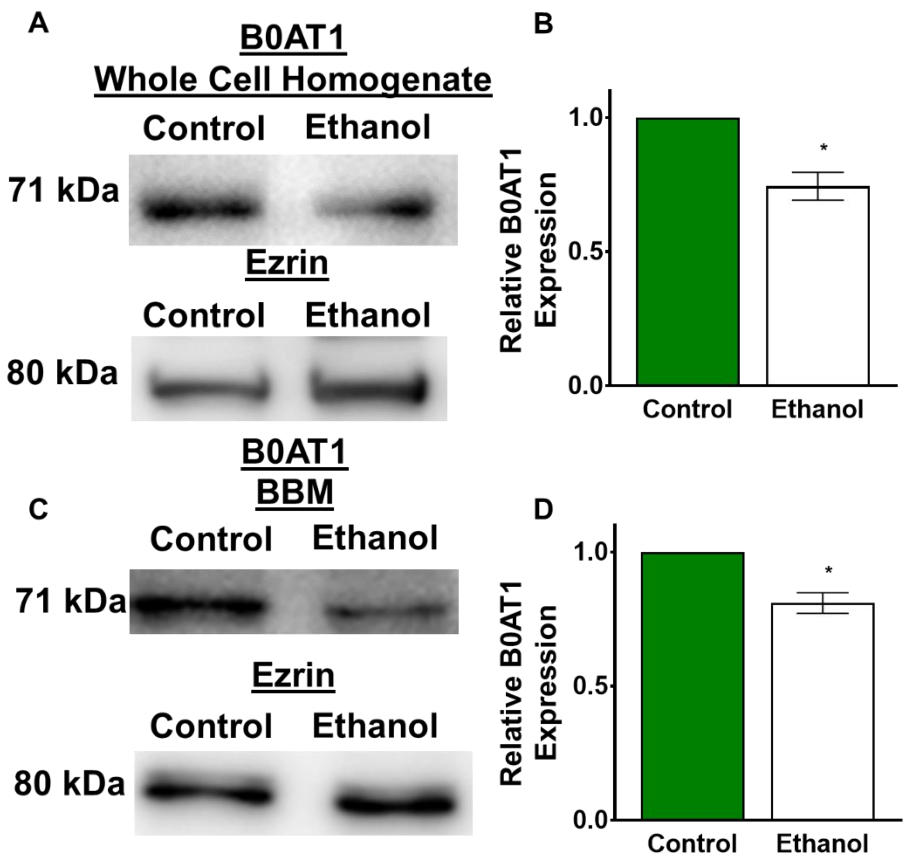


**Figure 11: Ethanol decreased the protein expression of B0AT1 in IEC-18 cells. A)** Western blot analysis of B0AT1 protein expression in the whole cell homogenates from control and ethanol-treated cells. The blots show a representative sample used for densitometric quantification. **B)** Densitometric quantification of B0AT1 protein expression was significantly decreased in whole cell homogenates after 1-hour exposure of ethanol (*, $p<0.05$, n=6). **C)** Western blot analysis of BBM B0AT1 protein expression from control and ethanol-treated cells. The blots show a representative sample used for densitometric quantification. **D)** Densitometric analysis of B0AT1 protein expression was significantly decreased in the BBM fraction after 1-hour exposure of ethanol (*, $p<0.05$, n=4). Error bars represent the SEM. Blots were normalized with an anti-ezrin antibody to assure equivalence of loading.

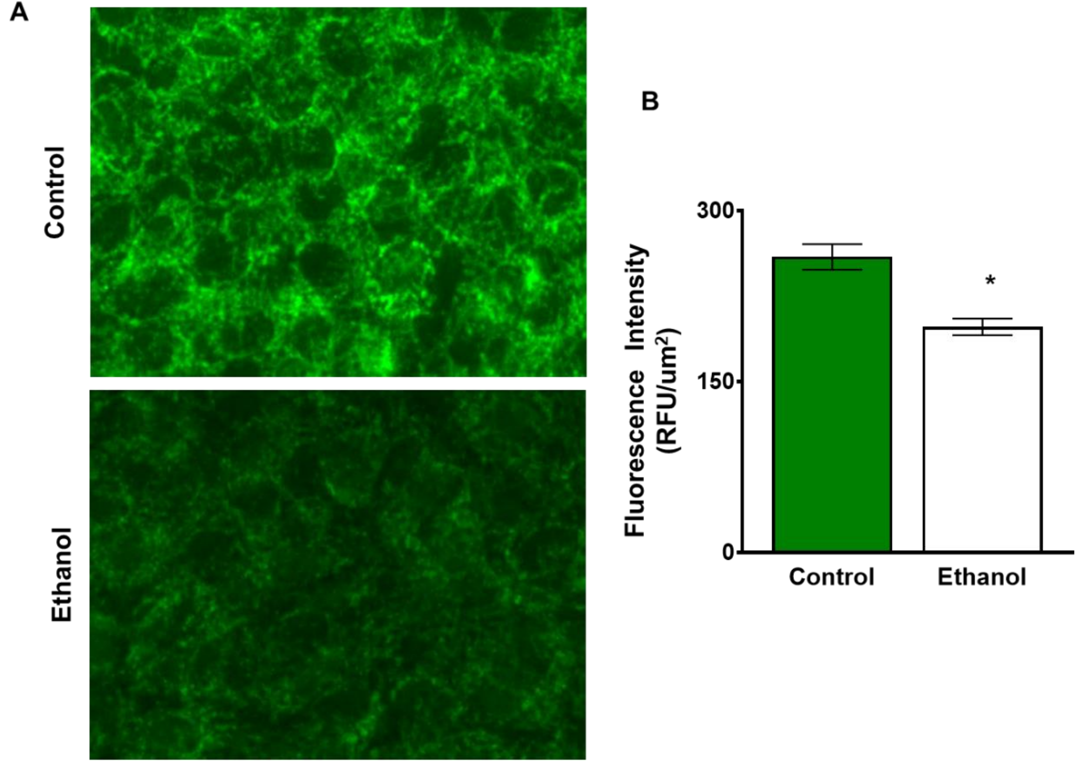


**Figure 12: One hour of ethanol significantly decreased B0AT1 immunofluorescence intensity**. **A)** The pictures are a representative image taken at the 60X objective. The green stain represents B0AT1. **B)** One hour of ethanol significantly decreased B0AT1 immunofluorescence intensity in IEC-18 cells (*, $p<0.05$, n=4). Each sample is an average of 5 different images obtained using a 20X objective. Error bars represent the SEM.

**The effect of PKC-silencing on glutamine absorption in IEC-18 cells exposed to ethanol**

PKC has been shown to affect nutrient co-transport in IEC-18 cells (Arthur and Sundaram 2014). Furthermore, the function of PKC has been shown to be altered in response to ethanol as well (Stubbs and Slater 1999). Therefore, in order to elucidate a possible mechanism of action for ethanol on B0AT1, PKC expression was selectively silenced in IEC-18 cells. In PKC siRNA-transfected IEC-18 cells, ethanol does not affect Na-dependent glutamine absorption (Figure 13; 1174.7±47.1 pmol/mg protein•2 min in control vs 732.7±118.9 in ethanol-

treated cells at 1-hour, $p<0.05$; 1147.7 pmol/mg protein•2 min in control+siRNA vs 1252.3±77.9 in ethanol-treated+siRNA cells at one hour, $p>0.05$, n=3) which indicated that PKC is likely important in the ethanol-mediated inhibition of B0AT1.

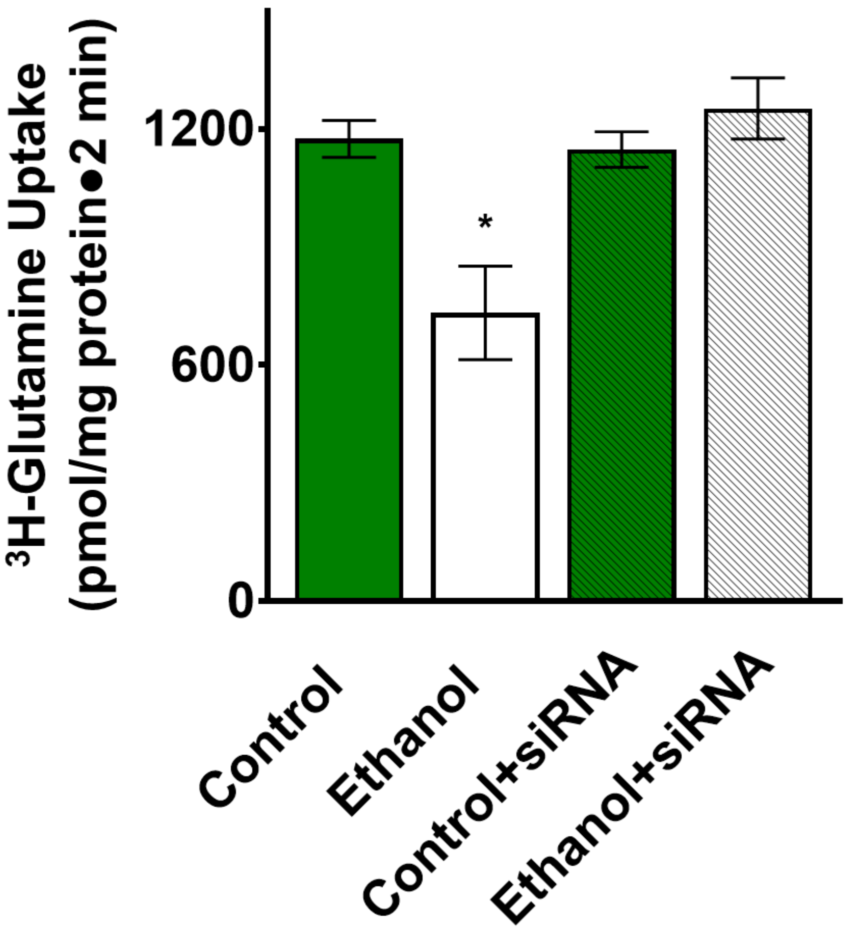


**Figure 13: Transfection with PKC-siRNA in IEC-18 cells reverses the ethanol-mediated inhibition of sodium-dependent glutamine absorption (*, p<0.05, n=3).**

**Discussion**

Chronic alcoholics are commonly malnourished, which is in part due to a suboptimal diet, but may also be due to ethanol-mediated decreased absorption of various vitamins in the small intestine including thiamine (Hoyumpa, Breen et al. 1975, Hoyumpa 1980, Hoyumpa,

Patwardhan et al. 1981), folate (Hamid, Wani et al. 2007, Thakur, Rahat et al. 2015), riboflavin (Subramanian, Subramanya et al. 2013), glucose (Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981), and minerals (Bode and Bode 2003). However, how ethanol affects the absorption of the amino acid glutamine has not yet been investigated. In this study, it was demonstrated that administration of a moderate dose of ethanol, equivalent to a 0.04% BAC, significantly decreased sodium-dependent glutamine uptake by affecting the B0AT1 co-transporter directly. This is the first report of a decrease in glutamine absorption in response to ethanol.

In this study, we showed that a moderate dose of ethanol decreases sodium-dependent glutamine absorption in IEC-18 cells by decreasing the activity of the primary means of assimilation of glutamine, namely, the Na-glutamine co-transporter B0AT1. The decrease in B0AT1 was not secondary to diminished cell viability. Further, the effect was secondary to ethanol and not its more toxic metabolite, acetaldehyde. At the cellular level, the inhibition of B0AT1 by moderate ethanol was secondary to both an effect at the level of the co-transporter in the BBM as well as secondary to diminished Na-extruding capacity of the cell. At the level of the co-transporter, the mechanism of inhibition of B0AT1 was secondary to a decrease in the maximal rate of uptake of glutamine ($V_{max}$) without a change in the affinity of the co-transporter for glutamine. Molecular studies demonstrated that BBM levels of B0AT1 were reduced by moderate ethanol. Finally, this inhibition of B0AT1 by ethanol appears to be mediated by the PKC pathway in intestinal epithelial cells.

In this study, moderate ethanol decreased the protein expression of B0AT1 in whole cell homogenate and in the BBM. Similar to our study, Subramanya and colleagues have shown that the protein expression levels of vitamin co-transporters are decreased by ethanol. Thiamine co-

transport was also shown to be decreased along the jejunal BBM and BLM by a reduction in the protein and mRNA expression of thiamine transporter-1, but not thiamine transporter-2, after male Wistar rats were exposed to the Lieber-DeCarli ethanol liquid diet (36% calorically ethanol) for 2, 4, and 6 weeks (Subramanya, Subramanian et al. 2010). Furthermore, in another study, the vitamin $B_2$ (riboflavin) transporters 1 and 3 mRNA and protein expression were decreased when Wistar rats were fed a Lieber-DeCarli ethanol liquid diet for four weeks (Subramanian, Subramanya et al. 2013). The results of our present study are consistent with these previous reports and thus furthering the current knowledge on ethanol-dependent malnutrition.

This study demonstrated that there was a decrease in the Na-K-ATPase activity in IEC-18 cells exposed to moderate ethanol. The Na-K-ATPase is vital in establishing the sodium gradient necessary for all sodium-dependent nutrient co-transporters to function at optimal levels. By decreasing the activity of the Na-K-ATPase, ethanol may decrease BBM glutamine co-transport by affecting the sodium gradient necessary for this co-transporter to properly function. Other studies have investigated the effect of ethanol on Na-K-ATPase activity and found that acute doses of ethanol decreased the activity of the Na-K-ATPase, but chronic doses of ethanol increased its activity (Rodrigo and Thielemann 1997, Otis, Mitchell et al. 2008, Rasic-Markovic, Krstic et al. 2008). In all, while ethanol clearly has a wide range of effects on the Na-K-ATPase, the effect of moderate ethanol on the BLM Na-K-ATPase appears to have a role in regulating Na-dependent nutrient assimilation by enterocytes.

This study, for the first time, showed the unique regulation of the glutamine absorption by a moderate dose of ethanol, specifically through decreased protein expression of B0AT1 in IEC-18 cells. The intracellular regulation of this observation has yet to be fully elucidated. However,

initial mechanistic studies, using siRNA-transfected IEC-18 cells, demonstrated that PKC has a regulatory role in this process. It has previously been shown that PKCα can regulate the sodium alanine co-transporter ASCT1 in intestinal epithelial cells (Arthur and Sundaram 2014). PKC has also been shown to alter the function of the Na-K-ATPase (Bibert, Roy et al. 2008) and ion channels in response to ethanol in a wide variety of studies. Ion channels altered by ethanol via PKC include the metabotropic glutamate receptor, the BK-channel, and the GABA chloride channel (Stubbs and Slater 1999). Thus, in our study and in the literature, there is a clear link between ethanol, PKC, and Na-nutrient co-transporters. However, further studies are required to fully elucidate the mechanism of action between ethanol, B0AT1 and PKC.

In conclusion, the sodium-dependent glutamine co-transporter, B0AT1, was significantly reduced by moderate ethanol in intestinal epithelial cells. The mechanism of inhibition was due to a decrease in the number of BBM co-transporters as well as well as a diminished sodium gradient set by the Na-K-ATPase. Furthermore, the PKC pathway likely mediates this inhibition of B0AT1 by moderate ethanol which provides evidence that even moderate doses of ethanol affects important nutrient uptake pathways in intestinal epithelial cells which may contribute to alcohol-mediated malnutrition in alcoholics and has implications on the treatment of malnutrition in chronic alcoholics.

**Acknowledgements**

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**Conflicts of Interest**

The authors have declared that no conflicts of interest exist.

# CHAPTER 5

## THE ACUTE EFFECTS OF MODERATE ALCOHOL CONSUMPTION ON SODIUM-DEPENDENT GLUCOSE CO-TRANSPORT IN RAT INTESTINAL EPITHELIAL CELLS IN VITRO AND IN VIVO

Molly Butts, Soudamani Singh, Jennifer Haynes, Subha Arthur and Uma Sundaram•

Department of Clinical and Translational Sciences and Appalachian Clinical and Translational Science Institute, Joan C. Edwards School of Medicine, Marshall University, 1600 Medical Center Drive. Huntington, WV 25701, USA

•Address Correspondence to:

Uma Sundaram MD

Joan C Edwards School of Medicine,

Marshall University

1600 Medical Center Drive

Huntington, WV 25701

Phone: 304 691 1841

Email: sundaramu@marshall.edu

Running Head: Ethanol regulates sodium-dependent glucose co-transport

**Abstract**

Chronic alcohol use often leads to malnutrition. However, how the intestinal absorption of nutrients like glucose may be affected during moderate ethanol use has not been investigated. Glucose is absorbed via sodium-dependent glucose co-transport (SGLT1; *SLC5A1*) along the brush border membrane (BBM) of intestinal absorptive villus cells. Rat intestinal epithelial cells (IEC-18) were exposed to 8.64 mM ethanol over 1, 3, 6, and 12 hours. Sixteen-week-old Sprague Dawley rats were administered 2 g/kg ethanol over 1, 3, and 6 hours. Sodium-dependent $^{3}$H-*O*-methyl-*D*-glucose uptake was performed to determine SGLT1 activity. Na-K-ATPase activity was measured as a function of inorganic phosphate release. Protein expression was analyzed by Western blot analysis and immunohistochemical staining. Ethanol significantly decreased Na-dependent glucose absorption and Na-K-ATPase activity in enterocytes *in vitro* and *in vivo*. Kinetic studies showed that the mechanism of inhibition of Na-glucose co-transport was secondary to a decrease in the affinity ($1/K_m$) of the co-transporter for glucose both *in vitro* and *in vivo*. Western blots and immunohistochemistry further demonstrated unaltered SGLT1 levels after ethanol treatment. In conclusion, moderate ethanol significantly decreases glucose absorption in enterocytes *in vitro* and *in vivo*. The inhibition of SGLT1 is secondary to altered Na-gradient at the cellular level and secondary to diminished affinity of the co-transporter for glucose at the protein level in the BBM. These observations may, at least in part, explain the malnutrition associated with alcohol consumption.

## Introduction

Chronic alcohol use has many well documented negative consequences including an increased risk of liver disease, cirrhosis, cancer, high blood pressure and stroke (Rehm, Baliunas et al. 2010, Organization 2018). Perhaps the most well-known but least understood complication of chronic alcoholism is malnutrition. In fact, how even moderate alcohol consumption, a considerably more common occurrence, may affect nutrient absorption in the mammalian small intestine is not fully understood. According to the 2015 National Survey on Drug Use and Health, almost 90% of adults report alcohol consumption during their lifetime. Moderate alcohol use, defined as one standard alcoholic beverage per day for women and two for men, imparts surprising health benefits including decreased risk for heart disease, ischemic stroke, diabetes, and obesity (NIAAA, Prevention). Nevertheless, beneficial or deleterious, how ethanol may affect intestinal nutrient absorption is poorly understood.

Chronic use of alcohol leads to malnutrition in chronic alcoholics, in part due to suboptimal nutrient intake (Lieber 2003). Alcohol use decreases absorption of essential vitamins along the small intestine including vitamin B12, vitamin B6, vitamin C, and selenium (Bode and Bode 2003). It also decreases the absorption of vitamin B1 (thiamine) (Subramanya, Subramanian et al. 2010), vitamin B2 (riboflavin) (Subramanian, Subramanya et al. 2013), and folate (Hamid, Wani et al. 2007) at the level of the nutrient co-transporter in the brush border membrane (BBM) of enterocytes. While the effect of ethanol on vitamin assimilation has been studied more often, its effect on nutrient absorption is less well understood. This study, for the first time, investigates alcohol's effect on nutrient absorption; specifically, the effect of moderate ethanol on the Na-dependent glucose co-transporter, SGLT1, complementarily *in vitro* and *in vivo*.

Glucose is a primary fuel source for the body. The primary means of absorption in the mammalian small intestine for glucose is via the Na-dependent glucose co-transporter SGLT1 (*SLC5A1*). SGLT1 is localized in the BBM of the villus, but not crypt cells, in the mammalian small intestine. SGLT1 is a 14-transmembrane domain protein, which is mainly expressed in the intestine. It facilitates the movement of two sodium ions into the cell with one glucose molecule (2:1 ratio). The favorable sodium gradient for SGLT1 in villus cells is provided by the basolateral membrane (BLM) Na-K-ATPase. Additionally, there is emerging evidence that SGLT1 regulates the other primary Na-absorptive mechanism in intestinal enterocytes via the sodium-proton exchanger NHE3 (Coon, Kekuda et al. 2008), (Lehmann and Hornby 2016).

Once glucose is absorbed into enterocytes, it can be transported and stored in the body to be used in a variety of metabolic processes including the pentose phosphate pathway and the Krebs cycle to produce energy in the form of adenosine triphosphate (Lehmann and Hornby 2016). Dysregulation of glucose absorption along the small intestine can lead to a variety of metabolic diseases, including malnutrition (Lieber 2003).

Previous studies have investigated the role of ethanol on glucose absorption. Overall, glucose absorption was significantly decreased by ethanol in hamsters (Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981), rats (Cobb, Van Thiel et al. 1983, al-Balool and Debnam 1989, Kaur, Kaur et al. 1995), chickens (Yunus, Awad et al. 2011), and dogs (Money, Petroianu et al. 1990). However, none of these studies investigated the effect of moderate ethanol on the sodium-dependent glucose co-transporter, SGLT1.

In this study, we aim to investigate the effect of a moderate dose of ethanol on SGLT1 *in vitro* and *in vivo* to better understand alcohol-dependent malnutrition.

## Methods

### Cell culture

The immortalized non-malignant rat intestinal epithelial cell line IEC-18 (CRL-1589 American Type Culture Collection) was cultured in Dulbecco's modified Eagle Medium (DMEM) (high glucose 4.5 g/l, sodium bicarbonate 3.7 g/l) containing 2 mM *L*-glutamine, 10% vol/vol bovine fetal serum, 0.02% insulin, and 0.25%-hydroxybutyric acid in a humidified atmosphere of 10% $CO_2$ at 37°C. The medium was changed every 2 to 3 days. Cells were grown to 4 days post confluence as monolayers, only using cells between passages 5 and 20. Cells were treated with 8.64 mM ethanol (200 proof, Pharmco Apper), the equivalent of a 0.04% blood alcohol content (BAC) dosage, directly into fresh medium for 1, 3, 6, or 12 hours. Control-treated cells were exposed to the same volume of sterile water in fresh medium.

### Lactate dehydrogenase (LDH) assays

Control and ethanol-treated cells were tested with a colorimetric LDH assay kit (Abcam) as per kit instructions to determine cell viability.

### Trypan blue exclusions

Control and ethanol-treated cells were washed with pre-warmed 1X phosphate buffered saline (PBS), and then harvested using 0.05% trypsin in PBS for 5 minutes at 37°C. Cell suspensions were mixed 1:1 with trypan blue solution (MilliporeSigma), and the stained cells were immediately counted with a hemocytometer.

### Uptake studies in IEC-18 cells

Uptake studies were performed as previously described (Arthur, Coon et al. 2014) in IEC-18 cells. In brief, sodium-dependent uptakes were conducted using Na-HEPES buffer (47 mM KCl, 1 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 20 mM HEPES, 125 mM $CaCl_2$, and 130 mM NaCl;

pH 7.4). To exclude passive uptake from active transport processes, specific reaction mixtures were used. Glucose uptakes were performed with and without 1 mM phlorizin, an SGLT1 inhibitor, and 100 μM of cold *D*-glucose. Both reaction buffers contained 10 μCi $^{3}$H-3-*O*-methyl-*D*-glucose (OMG) and 10 mM OMG-glucose. The reaction was stopped with ice-cold Na-HEPES buffer containing 25 mM *D*-glucose. Na/H uptakes were performed using 10 μCi $^{22}$Na with and without 50 μM EIPA, a Na/H exchanger inhibitor. The reaction was stopped with ice-cold TMA-HEPES. All reactions were performed for exactly 2 minutes. The cells were then processed as previously described (Arthur, Singh et al. 2018) and measured in a scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA).

**Protein quantification**

Total protein was measured by the Bradford method, using the Bio-Rad DC protein assay kit (Hercules, CA) with bovine serum albumin (BSA) as a standard.

**Na-K-ATPase measurement**

Isolated villus cells from rats were homogenized and centrifuged at 8000 x g for 5 minutes. The Na-K-ATPase activity was measured as inorganic phosphate ($P_i$) formation in cellular homogenates as previously described (Forbush 1983) and activity was expressed as nanomoles of $P_i$ released per milligram protein per minute.

**Animal studies**

Thirteen to fifteen-week-old male Sprague Dawley (SD) rats (001) were purchased from Charles River and allowed to acclimate to their environment for at least 1 week. All rats had equal access to normal chow and water and were kept in a 12-hour light and dark cycle. All oral gavage procedures started between 7 and 10 AM when the rats had aged to 16 weeks. An oral intragastric gavage was used with manual restraint, and 2 g/kg ethanol or tap water was

administered to ethanol-treated rats or control-treated rats, respectively (Livy, Parnell et al. 2003). At the 3 and 6-hour time points, blood for plasma separation for BAC measurement was collected via tail nick. Rats in the 6-hour treatment group were administered a second dose of ethanol 3 hours after the first dosage to maintain the correct BAC. Animals were euthanized with excess $CO_2$, and a final blood sample for plasma separation was collected via cardiac puncture. BAC was measured using an AM1 Alcohol Analyzer (Analox) and the respective Analox kit instructions. All animal handling, treatment, and euthanasia were carried out according to a protocol approved by the Marshall University IACUC.

**Villus cell isolation**

Villus cells were isolated from control and ethanol-treated rats from the ileal small intestine by a $Ca^{+2}$ chelation technique as previously described (Sundaram, Knickelbein et al. 1991). Briefly, a 12-inch section of the distal small intestine was filled with a buffer containing 0.15 mM EDTA, 112 mM NaCl, 25 mM $NaHCO_3$, 2.4 mM $K_2PO_4$, 0.4 mM $KH_2PO_4$, 2.5 mM L-glutamine, 0.5 mM β-hydroxybutyrate, and 0.5 mM dithiothreitol, and gassed with 95% $O_2$ and 5% $CO_2$, pH 7.4 at 37°C. The intestine was incubated for 3 minutes and palpitated for 3 minutes to aid in cell dispersion. The resulting cell suspension was then used for whole cell uptakes, or phenyl-methyl sulfonyl fluoride was added and centrifuged at 100 x g for 3 minutes, and the cellular pellet was frozen immediately in liquid nitrogen and stored at -80°C for future use.

**Uptake studies in villus cells from rats**

Intact whole cell uptakes were conducted (100 mg wet weight) immediately following the villus cell isolation. Cells were washed and suspended in the 37°C Na-HEPES buffer described above. In room temperature vials, 100 µL of the previously described reaction mixtures were added to 100 µL of the cellular suspension. The uptake was halted at exactly 2 minutes with ice-

cold stop solution as described above. The mixture was filtered on 0.65 μm mixed cellulose esters membrane filters (DAWP; Millipore) and washed twice with ice-cold stop solution using vacuum filtration. Each filter was dissolved in 5 mL scintillation solution, and radioactivity was measured as above.

**Brush border membrane vesicle (BBMV) preparation and uptake**

BBMV from rat intestinal villus cells were prepared by $CaCl_2$ precipitation and differential centrifugation as previously described (Talukder, Kekuda et al. 2008, Arthur, Singh et al. 2018). The final BBMV was incubated for an hour in a sodium-free buffer at room temperature, and 5 μL of BBMV were incubated in 95 μL reaction buffer as described above. The reaction was arrested at 90 seconds and filtered on a 0.45 μm mixed cellulose esters membrane filters (HAWP; Millipore). Each filter was washed twice with ice-cold stop solution using vacuum filtration, then dissolved in scintillation fluid, and measured as above.

**Kinetics**

Sodium-dependent glucose uptakes were performed at 30 seconds at varying concentrations of glucose (0.1, 0.5, 1, 5, 10, 25, 75 and 100 mM) as described above. Uptake values were evaluated by Michaelis-Menten kinetics using a non-linear regression data analysis using Prism 7 software (GraphPad, San Diego, CA). Isolated rat villus cells were used to create BBMVs and kinetics were performed as above.

**Western blot studies**

Whole cell homogenates were prepared by first centrifuging cells at 8000 x g at 4°C for 5 minutes and solubilizing the resulting pellet in RIPA buffer containing protease inhibitors (Santa Cruz, CA). Cellular extracts in RIPA were then centrifuged at 8000 x g at 4°C for 5 minutes, and the supernatant was measured for protein content using a NanoDrop Spectrophotometer (Thermo

Scientific). Proteins from BBM preparations were solubilized and extracted in RIPA buffer as above. Proteins were separated on a 12% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, blocked in 5% BSA for IEC-18 samples or 5% milk for SD rat samples for 1 hour as previously described (Singh, Arthur et al. 2015, Singh, Arthur et al. 2018). Rabbit polyclonal primary antibodies against SGLT1 (Abcam 14686) were used at 1:500 dilution at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit secondary antibodies at 1:1000 dilution at room temperature for 1 hour were also used. ECL-TM Western Blotting Detecting Reagent detected the immobilized proteins. Luminescence was detected and analyzed by densitometry using a FluorChem M imager (ProteinSimple). All blots were stripped, and re-probed with a mouse monoclonal primary antibody against ezrin (MilliporeSigma MAB3822-C) at 1:1000 dilution as above, and proteins were normalized against the ezrin values.

**Immunocytochemistry (ICC)**

Control and ethanol-treated cells grown on glass coverslips were fixed with ice-cold methanol for 4 minutes. Non-specific binding of primary antibodies was blocked using 5% BSA and 0.1% Triton-X for 1 hour at room temperature. Cells were then washed in room temperature PBS, 3 times for 5 minutes each and incubated with SGLT1 primary antibody (Abcam 14686) at a 1:250 dilution for 1-hour. Cells were washed as before, then incubated with goat anti-rabbit Alexa Fluor 594 secondary antibody (Invitrogen, Eugene, OR) for 1 hour at a 1:500 dilution. The cells were washed again, mounted with Fluoroshield Mounting Medium with DAPI (Abcam) and imaged using a 20X objective on an EVOS FL Auto 2 microscope (Invitrogen) and quantified with AlphaView software version 3.4.0.0.

### Immunohistochemistry (IHC)

A half inch of terminal ileum was separated for IHC. The ileum was gently washed with room temperature PBS, then flash frozen using liquid nitrogen and stored at -80°C until processing. Upon use, samples were quickly placed in Tissue Plus O.C.T. Compound (Fisher HealthCare) and frozen using dry ice in methanol to avoid thawing the sample. Samples were sectioned 3 µm thick at -20°C using a cryostat. Sections were fixed in -20°C methanol for 30 sec and processed with the same blocking, primary and secondary antibodies used above. Tissue sections were mounted, imaged and analyzed as above.

### Statistical analysis

Data were computed in triplicate and mean ± standard error were evaluated. P-values were derived by unpaired t-test. In cases of multiple time points, multiple hypothesis testing was addressed with a Bonferroni correction using Prism 7 software (GraphPad, San Diego, CA). A p-value of less than 0.05 was considered statistically significant. To check for the robustness of the data sets, non-parametric methodology was used. Each number (n) for any set of experiments refers to cell preparations from different passages.

## Results

### Cell viability in response to a moderate dosage of ethanol in IEC-18 cells

Ethanol has been shown to be toxic to cells at high concentrations (Goldstein 1987). To ensure that our dosage of moderate ethanol (8.64 mM or the equivalent of a 0.04% BAC), was not cytotoxic, LDH and trypan blue exclusion experiments were performed. This dose of ethanol did not cause cell damage or death over 1, 3, 6, and 12 hours (Figure 14A, B). In the LDH assay, ethanol-treated cells did not significantly differ from controls (ethanol-treated cells were 98.6±0.6% of controls, $p>0.05$, $n=4$). In trypan blue assays, ethanol-treated cells did not

significantly differ from controls (98.5±1.0% in controls vs 97.6±0.3% in ethanol-treated cells at 1 hour, p>0.05, n=4). Cell survival between 95-100% are considered within normal physiological parameters.

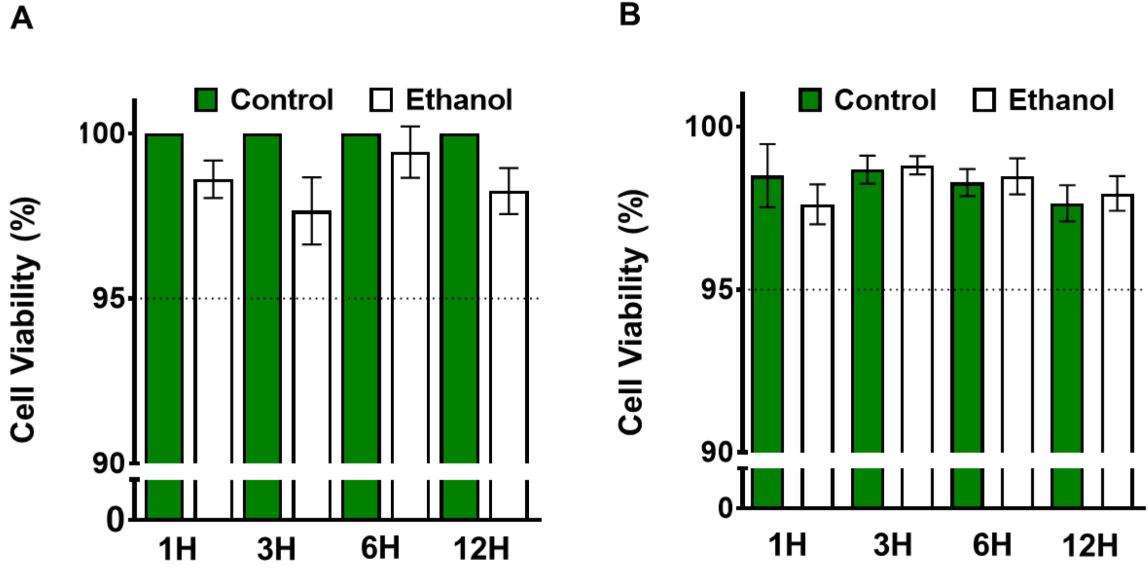


**Figure 14. Moderate ethanol (8.64 mM) does not significantly decrease cell viability in IEC-18 cells. A)** Ethanol does not cause significant cell death in IEC-18 cells using an LDH assay. For LDH assays, ethanol-treated cells were normalized to control-treated cells (1H: p=0.20; 3H: p=0.24; 6H: p=2.00; 12H: p=0.20; n=4). **B)** Ethanol does not cause significant cell death in IEC-18 cells using trypan blue assays (1H: p=1.07; 3H: p=3.17; 6H: 2.15; 12H: p=3.37; n=4). Error bars represent the standard error of the mean (SEM).

**Effect of ethanol on sodium-dependent glucose uptake in IEC-18 cells**

To determine if moderate ethanol affects glucose absorption, $^{3}$H-OMG uptakes were conducted in IEC-18 cells exposed to 1, 3, 6 and 12 hours of ethanol. Ethanol significantly decreased sodium-dependent glucose absorption at all time points (Figure 15; 502±27.3 pmol/mg protein•2 min in control vs 243±13.5 in ethanol-treated cells at 1-hour, p<0.01, n=6).

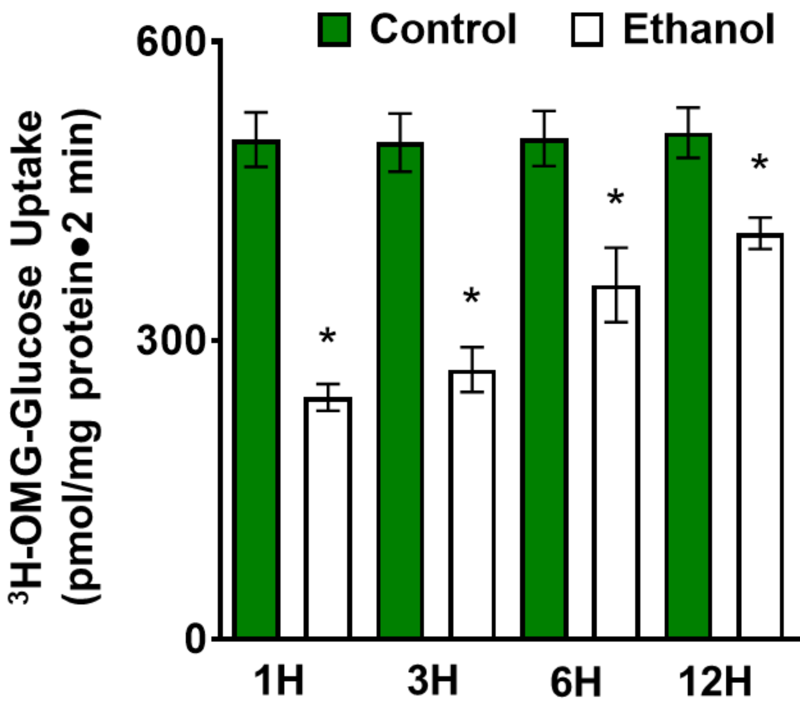


**Figure 15: Moderate ethanol decreased glucose absorption in IEC-18 cells.** Na-dependent glucose uptake was determined as a function of phlorizin-sensitive $^{3}$H-OMG uptakes in the presence and absence of moderate ethanol (*, 1H: $p<0.01$; 3H: $p<0.01$; 6H: $p=0.04$; 12H: $p=0.03$; n=6). Error bars represent the SEM.

**Effect of ethanol on Na-K-ATPase activity in IEC-18 cells**

The Na-K-ATPase located on the BLM provides the transcellular sodium gradient necessary for sodium-dependent nutrient co-transporters. The Na-K-ATPase activity was significantly inhibited by moderate ethanol at all time points (Figure 16; 22.1±1.76 nmol/mg protein•min in control vs 8.17±0.95 in ethanol-treated cells at 1-hour, $p<0.01$, n=6).

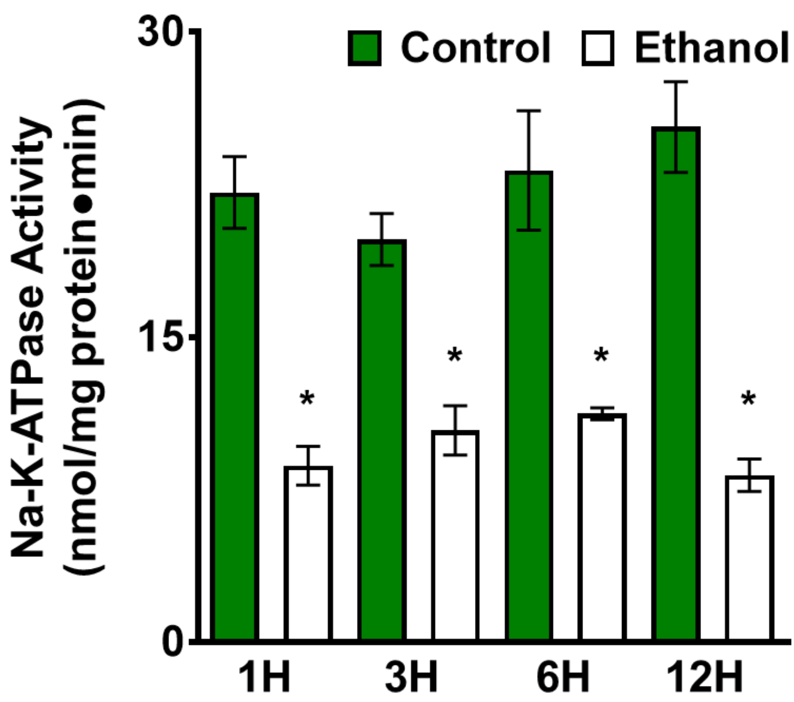


**Figure 16: Moderate ethanol significantly decreased Na-K-ATPase activity in IEC-18 cells.** The Na-K-ATPase activity was determined as a function of $P_i$ release in the presence and absence of moderate ethanol (*, 1H: p<0.01; 3H: p<0.01; 6H: p=0.01; 12H: p<0.01; n=6). Error bars represent the SEM.

**Effect of acetaldehyde on glucose uptake in IEC-18 cells**

Ethanol is metabolized into acetaldehyde, which can cause cell damage by forming adducts with other molecules, like lysine, at concentrations as low as 5 µM (Fisher, Swaan et al. 2010). Thus, it was determined how much acetaldehyde was produced in response to one hour of ethanol treatment in IEC-18 cells using a commercially available kit (Bioassays systems). Approximately 11 µM acetaldehyde was measured in IEC-18 cells following one hour of ethanol exposure (data not shown). Then, in a separate experiment, acetaldehyde was added directly to the cell culture medium of IEC-18 cells for one hour (15 µM) to determine if acetaldehyde affects SGLT1 activity. There was no change in sodium-dependent glucose uptake following

exposure to 15 µM acetaldehyde (Figure 17; 539±50.2 pmol/mg protein•2 min in control vs 533±52.9 in acetaldehyde-treated cells at 1-hour, $p>0.05$, n=4).

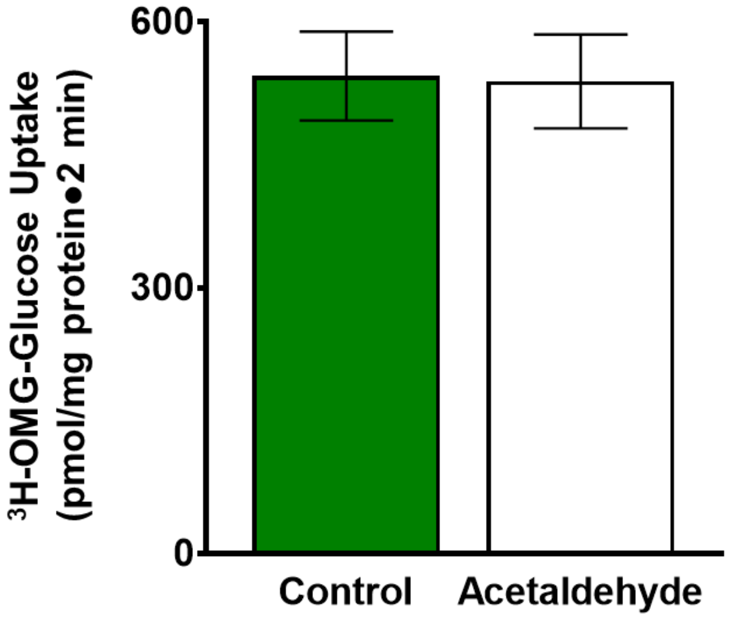


**Figure 17: Acetaldehyde (15 µM) did not affect glucose absorption in IEC-18 cells.** Na-dependent glucose uptake was determined as a function of phlorizin-sensitive $^{3}$H-OMG uptakes in the presence and absence of acetaldehyde (n=4). Error bars represent the SEM.

**Effect of ethanol on Na/H exchange in IEC-18 cells**

Sodium-dependent glucose co-transport and Na/H exchange are the two major sodium-absorptive pathways located on the BBM of intestinal epithelial cells. Further, the Na-K-ATPase maintains the cellular sodium concentration which we established was decreased by ethanol in IEC-18 cells. Thus, to investigate if ethanol also affects BBM Na/H exchange in IEC-18 cells, we conducted $^{22}$Na-uptakes. We demonstrated that moderate ethanol did not change Na/H exchange in IEC-18 cells (Figure 18; 1120±18.3 pmol/mg protein•2 min in control vs 1104±41.9 in ethanol-treated cells at 1-hour, $p>0.05$, n=4) which indicates that ethanol's effect on Na-absorptive pathways in IEC-18 cells is selective.

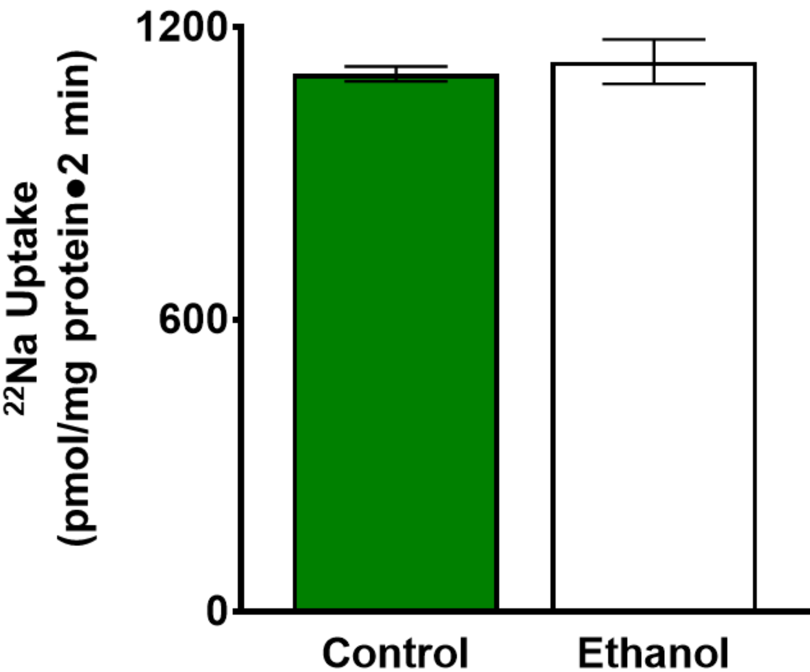


**Figure 18: Ethanol did not alter Na/H exchange in IEC-18 cells.** Na/H uptake was determined as a function of $^{22}$Na uptake in the presence and absence of moderate ethanol (n=4). Error bars represent the SEM.

**Effect of ethanol on the kinetic parameters of sodium-dependent glucose co-transport in IEC-18 cells**

Kinetic studies were conducted to determine the mechanism of inhibition of sodium-dependent glucose co-transport by moderate ethanol. The mechanism of inhibition of Na-glucose co-transport in IEC-18 cells by ethanol was secondary to a decrease in the affinity of the co-transporter for glucose without a change in the maximal rate of uptake of the co-transporter ($V_{max}$), suggesting a decreased affinity of SGLT1 to glucose, rather than a reduction in co-transporter number (Table 5).

| | $V_{max}$ (nmol/mg protein•30sec) | $K_m$ (mM) |
|---|---|---|
| **Control – IEC-18** | 4.9±0.2 | 5.6±0.5 |
| **Ethanol – IEC-18** | 5.1±0.3 | 14.0±1.0* |
| **Control – SD rats** | 5.4±0.6 | 12.4±0.3 |
| **Ethanol – SD rats** | 5.3±0.5 | 15.3±0.4* |

**Table 5: Effect of ethanol on the kinetic parameters of sodium-dependent glucose co-transport in rat intestinal epithelial cells in vitro and in vivo.** Na-dependent glucose uptake was determined by varying concentrations of glucose using phlorizin-sensitive $^{3}$H-OMG uptakes in the presence and absence of moderate ethanol. Uptake for all concentrations was conducted at 30 seconds. In IEC-18 cells, the affinity of the sodium-dependent co-transporter SGLT1 was decreased as represented by an increase in $K_m$ when exposed to 1 hour of ethanol (*, $p<0.01$, n=4). In SD rats, the affinity of the sodium-dependent co-transporter SGLT1 was decreased, represented by an increase in $K_m$, when exposed to 1-hour of ethanol (*, $p<0.01$, n=3).

**Quantification of SGLT1 protein expression following exposure to moderate ethanol in IEC-18 cells**

Western blot analysis of SGLT1 protein was performed on whole cell homogenates and BBM preparations. Protein expression of SGLT1 in whole cell homogenates was not significantly altered in IEC-18 cells after one-hour exposure to ethanol, as quantified by densitometry (Figure 19 A-B; ethanol-treated cells were 0.98 of control, $p>0.05$, n=4). Similarly, there was no difference in SGLT1 protein levels in IEC-18 cell BBM between control and ethanol-treated IEC-18 cells (Figures 19 C-D; ethanol-treated cells were 1.04 of control, $p>0.05$, n=4).

In addition, ICC studies were performed in control and ethanol-treated cells exposed to 1 and 12 hours of ethanol. Consistent with Western blot data, SGLT1 immunofluorescence intensity was unchanged in cells exposed to ethanol (Figure 20 A-B).

These data, along with the kinetic analysis above, demonstrate that the mechanism of inhibition of ethanol-mediated sodium-dependent glucose co-transport in IEC-18 cells is via a change in the affinity of SGLT1 for glucose and not through a change in the protein expression.

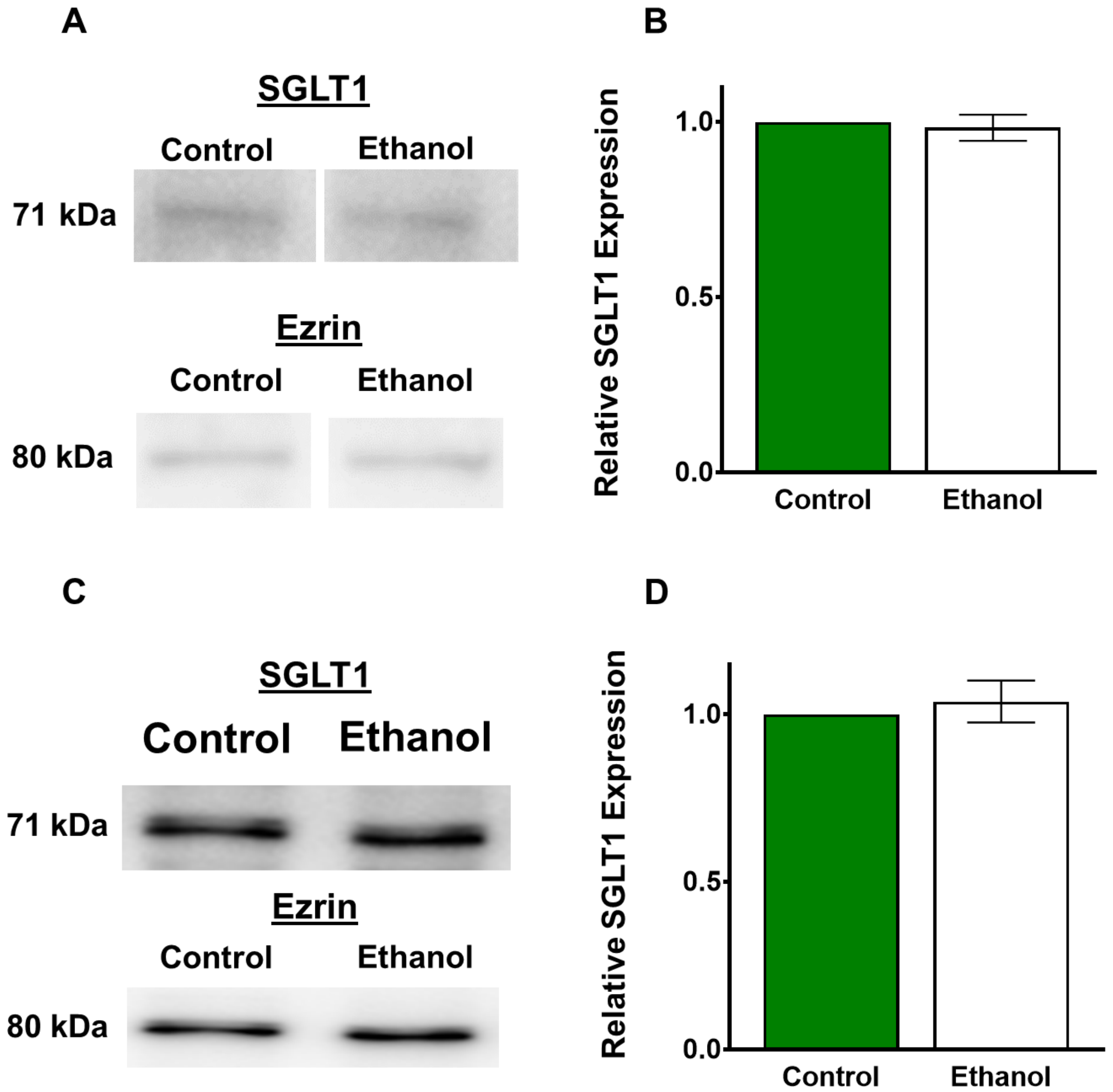


**Figure 19: Ethanol did not change SGLT1 protein expression in IEC-18 cells.** **(A)** Western blot analysis of SGLT1 protein expression in whole cell homogenates from control and ethanol-treated cells. The blots show a representative sample used for densitometric quantification (n=4). **(B)** Densitometric quantification of SGLT1 protein expression was not significantly changed in whole cell homogenates after 1-hour exposure of ethanol (n=4). **(C)** Western blot analysis of BBM protein expression of SGLT1 from control and ethanol-treated cells. The blots show a representative sample used for densitometric quantification. **(D)** Densitometric analysis of SGLT1 protein expression was not significantly altered in the BBM fraction after 1-hour

exposure to ethanol (n=4). Error bars represent the SEM. Blots were normalized with an ezrin antibody to assure equivalence of loading.

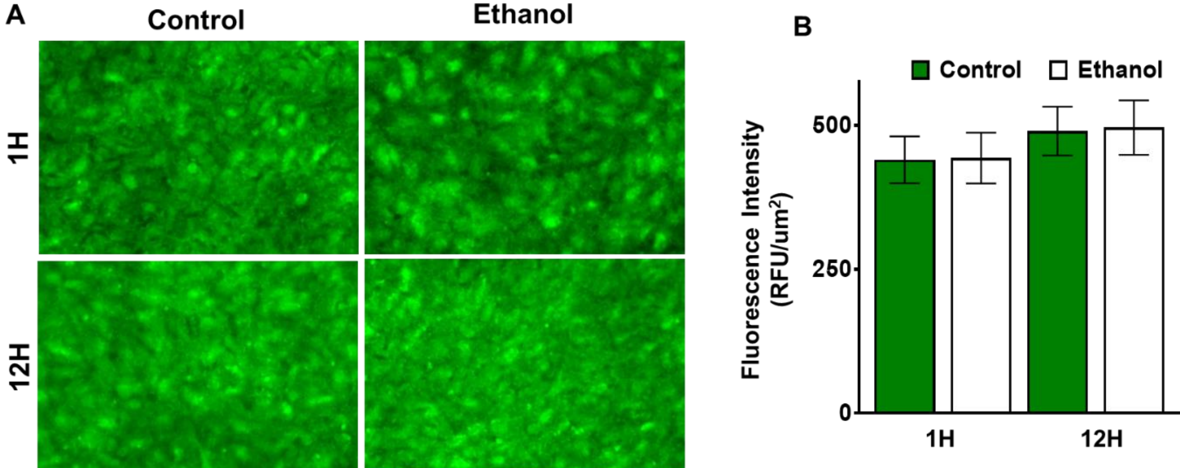


**Figure 20: SGLT1 expression in IEC-18 cells after exposure to ethanol. (A)** The effect of ethanol on SGLT1 expression in IEC-18 cells imaged with the 20X objective. **(B)** One hour of ethanol did not significantly change SGLT1 immunofluorescence intensity in IEC-18 cells (1H: p=1.98; 12H: p=1.80; n=4). Each sample is an average of at least 5 different images obtained using a 20X objective. The error bars represent the SEM.

**Effect of a short-term oral gavage of ethanol in SD rats**

To determine the *in vivo* effect of moderate ethanol consumption on SGLT1, SD rats were utilized. Male rats, 16 weeks old, were administered ethanol by intragastric gavage. The body weights of the rats at the time of gavage did not differ (Figure 21A). The BAC of the rats was measured at the time of euthanasia or, in the longer time points, one hour after ethanol administration. The rats achieved the equivalent of 0.04% BAC (Figure 21B).

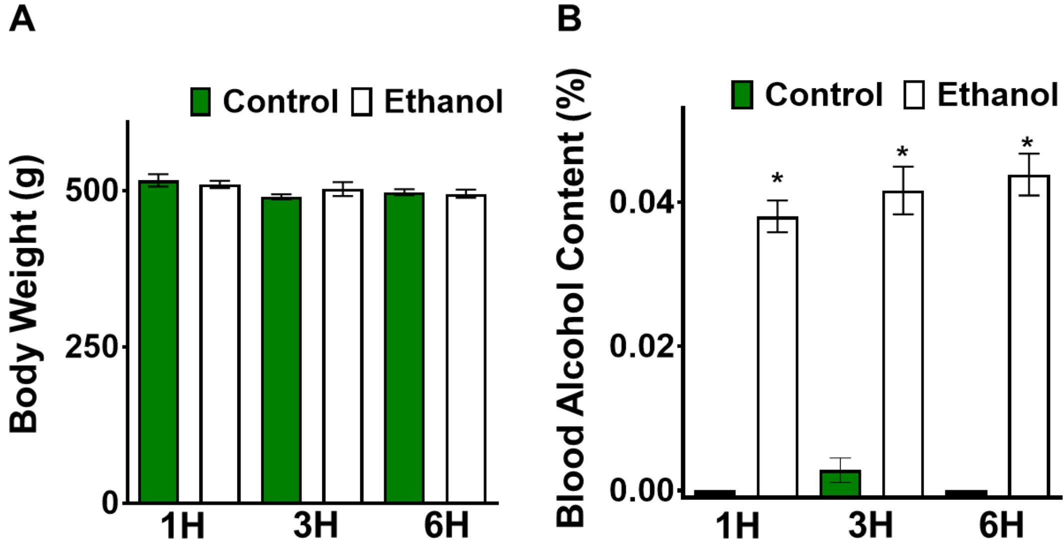


**Figure 21: Establishment of the Sprague Dawley rat model using an oral gavage of ethanol.** **(A)** Body weights of 16-week old SD rats before oral gavage did not differ (1H: p=1.80; 3H: p=0.99; 6H: p=2.31; n=4). **(B)** The administration of 2 g/kg ethanol achieved a BAC of 0.04% in SD rats (*, 1H: p<0.01; 3H: p<0.01; 6H: p<0.01; n=4). The error bars represent the SEM.

**Effect of ethanol on Na-glucose co-transport in intestinal epithelial cells in vivo**

To determine if moderate ethanol affects glucose absorption *in vivo*, $^{3}$H-OMG uptakes were conducted in villus cells isolated from rats exposed to 1, 3, and 6 hours of ethanol. Sodium-dependent glucose absorption was significantly decreased at all time points in ethanol-administered rats (Figure 22A; 122±8.67 pmol/mg protein•2 min in control vs 71.0±3.35 in ethanol-treated rats at 1-hour, p<0.01, n=4).

**Effect of ethanol on Na-K-ATPase activity in vivo**

Intestinal epithelial cell Na-K-ATPase activity, as measured by the release of inorganic phosphate, was also decreased in ethanol-treated rats at all time points *in vivo* (Figure 22B; 22.1±1.67 nmol/mg protein•min in control vs 9.3±0.72 in ethanol-treated rats at 1-hour, p<0.05, n=4).

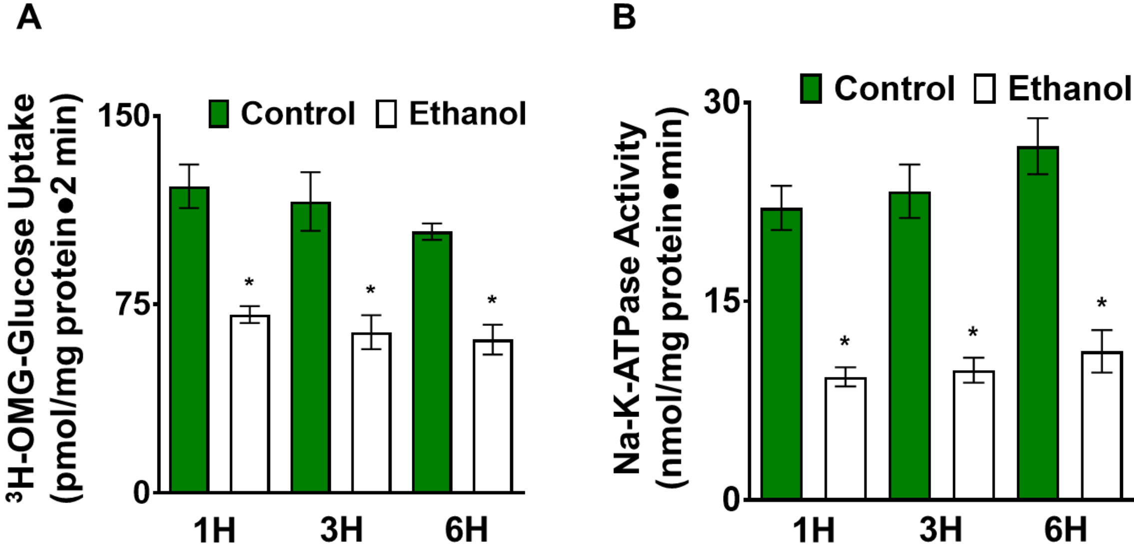


**Figure 22: An oral gavage of ethanol significantly decreased glucose absorption and Na-K-ATPase activity in SD rats. (A)** Whole villus cell phlorizin-sensitive 3H-OMG uptakes decreased in SD rats exposed to ethanol (*, 1H: $p<0.01$; 3H: $p=0.03$; 6H: $p<0.01$; n=4). **(B)** Ethanol decreased Na-K-ATPase activity in SD rats (*, 1H: $p<0.01$; 3H: $p=0.01$; 6H: $p=0.01$; n=4). The error bars represent the SEM.

**Effect of ethanol on sodium-dependent glucose BBMV uptakes in SD rats**

To determine if ethanol has a direct effect on Na-glucose co-transport in the BBM of the villus cells, BBMVs were studied. Na-dependent glucose absorption was significantly decreased in the BBMVs from ethanol-treated animals (Figure 23; 3.04±0.26 pmol/mg protein•90 secs in control vs 1.28±0.19 in ethanol-treated rats, $p<0.01$, n=4).

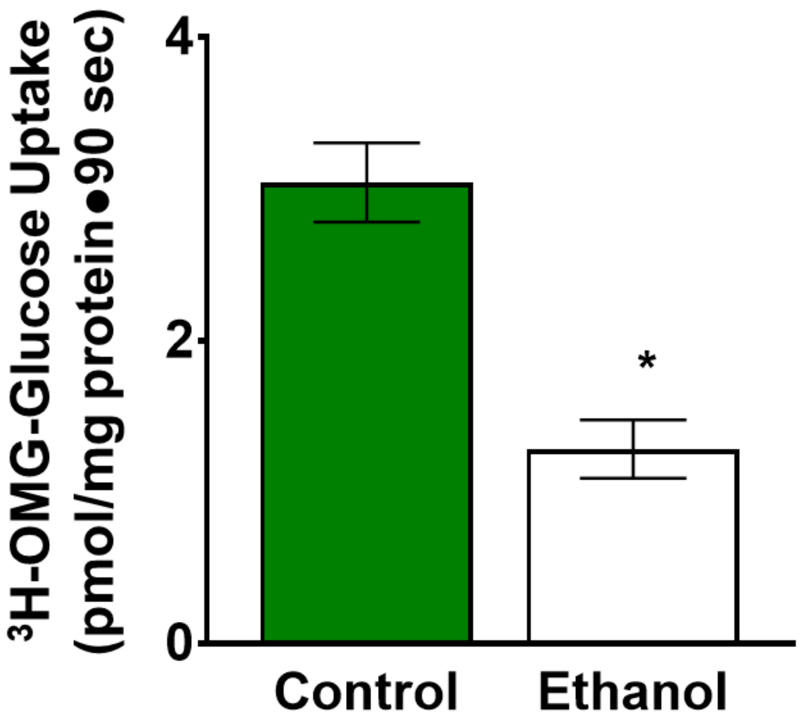


**Figure 23: An oral gavage of ethanol significantly decreased BBMV $^3$H-glucose uptake in SD rats.** Na-dependent glucose uptake was determined as a function of phlorizin-sensitive $^3$H-OMG uptakes in the presence and absence of moderate ethanol (*, $p<0.01$, $n=4$). The error bars represent the SEM.

**Effect of ethanol on the kinetic parameters of sodium-dependent glucose co-transport in SD rats**

Kinetic studies were conducted in BBMVs from rats exposed to ethanol for one hour. The mechanism of inhibition of sodium-dependent glucose co-transport *in vivo* was secondary to a decrease in the affinity of the co-transporter for glucose ($1/K_m$) without a change in the maximal rate of uptake of the co-transporter ($V_{max}$) (Table 5), suggesting no reduction in SGLT1 co-transporter number, as seen in the previous *in vitro* studies.

**Effect of in vivo ethanol consumption on SGLT1 protein expression in SD rats**

To determine if moderate ethanol affects SGLT1 protein expression, Western blot analysis was performed on whole cell homogenates. SGLT1 levels in the whole cell

homogenates were not significantly altered in SD rats after one-hour exposure of ethanol, as quantified by densitometry (Figure 24 A-B; ethanol-treated cells were 1.07 of control, $p>0.05$, $n=4$). Since SGLT1 is a BBM protein, Western blot analysis of SGLT1 protein was performed in the BBM as well. SGLT1 BBM immune reactive protein levels were not significantly altered in SD rats after one-hour exposure of ethanol as quantified by densitometry (Figure 24 C-D; ethanol-treated cells were 1.06 of control, $p>0.05$, $n=4$).

Next, IHC studies were performed in the small intestine of control and ethanol-treated rats (Figure 25A). Quantification of immunofluorescence intensity showed that control-treated rats did not significantly differ from ethanol-treated rats (Figure 25B). Furthermore, Na-K-ATPase immunofluorescence intensity also did not change between the control and ethanol-treated rats (Figure 25C).

Taken together, these molecular and kinetic data showed that ethanol, at a dosage equivalent to a BAC of 0.04%, significantly inhibited SGLT1 through a decrease in the affinity of the co-transporter without a change in the number of co-transporters *in vivo* which is directly comparable to the previous *in vitro* studies in IEC-18 cells.

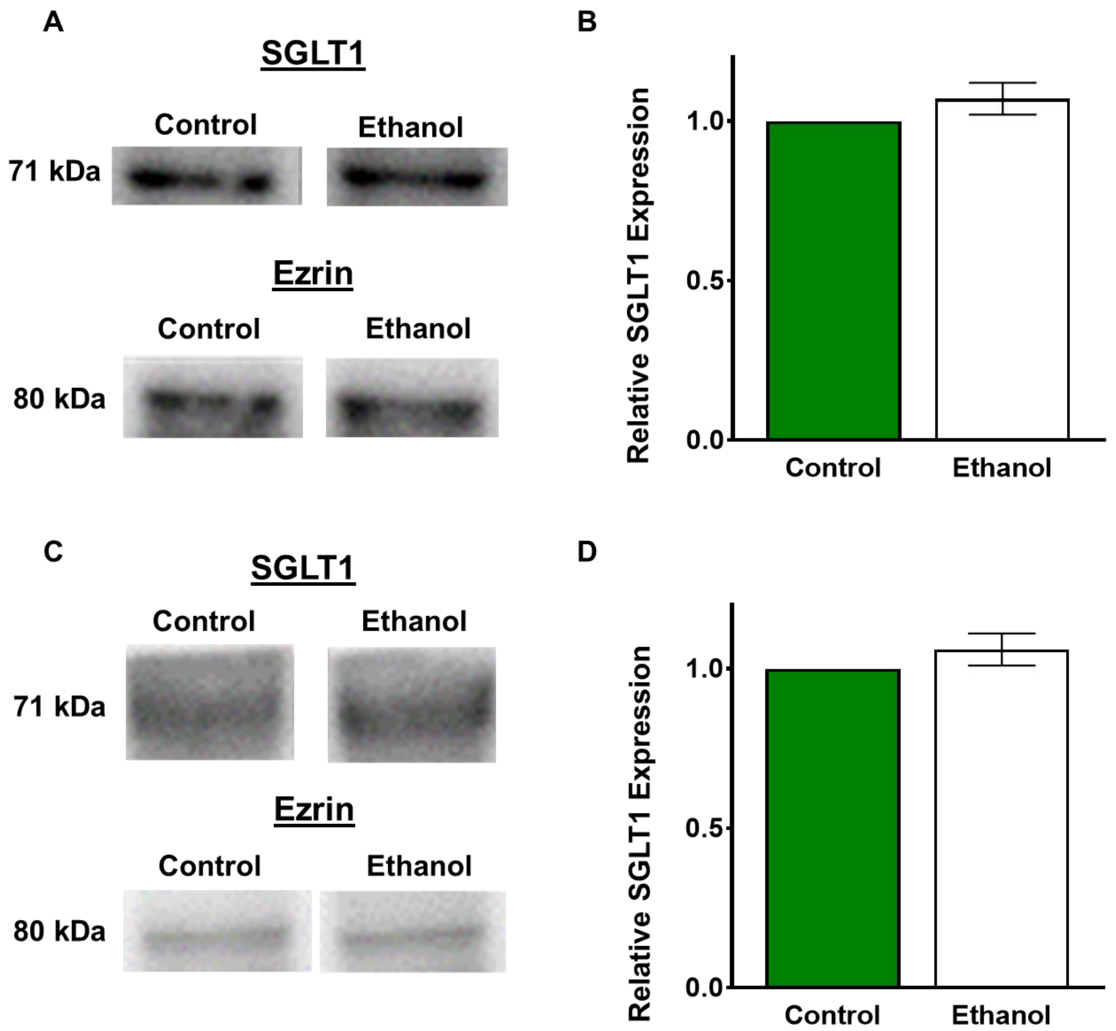


**Figure 24: An oral gavage of ethanol in SD rats did not change protein expression levels of SGLT1. (A)** Western blot analysis of SGLT1 protein expression in whole cell homogenates from control and ethanol-treated SD rats. The blots show a representative sample used for densitometric quantification. Separated control and ethanol samples were run on the same membrane but were not located next to each other. **(B)** Densitometric quantification of SGLT1 protein expression was not significantly changed in the whole cell homogenate fraction after 1-hour exposure to ethanol (n=4). **(C)** Western blot analysis of SGLT1 protein expression in the BBM from control and ethanol-treated SD rats. The blots show a representative sample used for densitometric quantification. **(D)** Densitometric quantification of SGLT1 protein expression was not significantly altered in the BBM fraction after 1-hour exposure of ethanol (n=4). The error bars represent the SEM. Blots were normalized with an anti-ezrin antibody to assure equivalence of loading. All control and ethanol-treated samples were run on the same membrane.

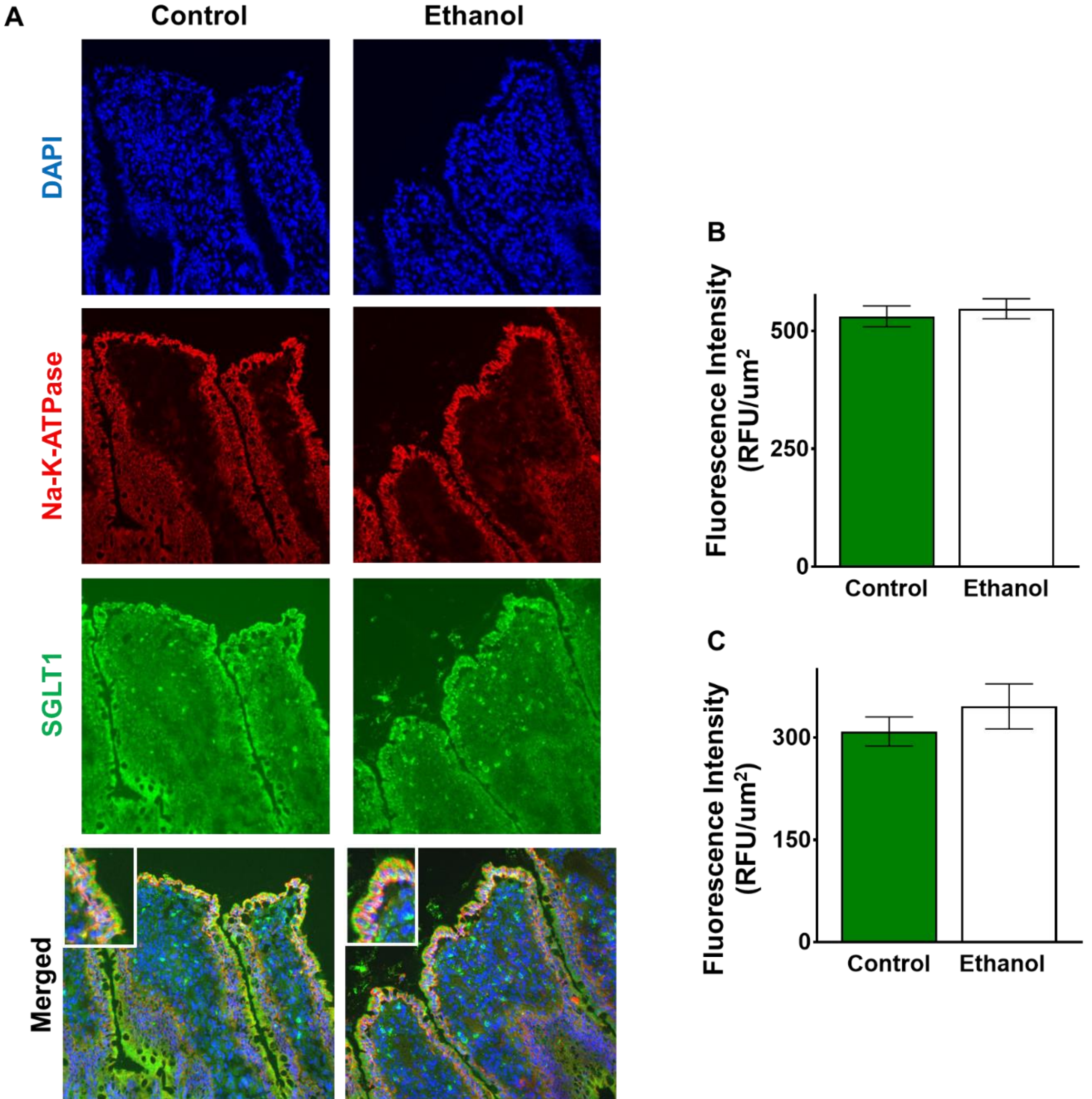


**Figure 25: An oral gavage of ethanol in SD rats did not change SGLT1 or Na-K-ATPase expression.** **(A)** SGLT1 and Na-K-ATPase expression in SD rats after exposure to 1-hour of ethanol. The blue stain represents DAPI, the green stain represents SGLT1 and the red stain represents the Na-K-ATPase. SGLT1 is localized to the apical side, or BBM, of the cell while the Na-K-ATPase is localized to the BLM. **(B)** One hour of ethanol did not significantly change SGLT1 immunofluorescence intensity in SD rats (n=4). **(C)** One hour of ethanol did not significantly change Na-K-ATPase immunofluorescence intensity in SD rats (n=4). Each sample is an average of at least 5 different images obtained using a 20X objective. The error bars represent the SEM.

**Discussion**

Previous studies investigating the effect of ethanol on nutrition showed that ethanol could decrease the absorption of a wide variety of vitamins (Bode and Bode 2003). Similar to our study, others have also investigated the effect of ethanol on vitamin absorption at the level of their respective co-transporters (Hoyumpa, Breen et al. 1975, Romanoff, Ross et al. 2007, Subramanya, Subramanian et al. 2010, Guo, Wang et al. 2013, Subramanian, Subramanya et al. 2013). While there have been studies pertaining to nutrition and ethanol, no other study has investigated the effect of moderate ethanol on the sodium-dependent glucose co-transporter SGLT1 *in vitro* and *in vivo*. SGLT1 is important because it transports glucose, an essential nutrient for intestinal cell and whole-body metabolism. Thus, the goal of this study was to investigate the effect of a moderate dose of ethanol, equivalent to a BAC of 0.04%, on Na-dependent glucose absorption in enterocytes *in vitro* and *in vivo*.

Studies conducted using the same dosage of moderate ethanol (8.64 mM) in neuronal-like pheochromocytomas cells showed no cell death after 12 hours of continuous ethanol exposure (Luo, West et al. 1999). This directly supports our cell viability results using an LDH assay and trypan blue exclusion. This moderate dosage of ethanol did not significantly alter cell viability in IEC-18 cells.

This study showed that moderate ethanol affects glucose assimilation via SGLT1 in intestinal epithelial cells. When exposed to a moderate dose of ethanol both *in vitro* and *in vivo,* the effects of ethanol on SGLT1 are comparable. Previous studies conducted using inverted intestinal sacs showed that an extremely high dosage of ethanol (450 mM) decreased glucose absorption in the jejunum of hamsters (Dinda, Beck et al. 1975, Dinda and Beck 1977). This study did not determine if SGLT1 was specifically affected by ethanol, but the researchers

hypothesized that there is most likely interference of glucose absorption at the co-transporter level, which directly supports our findings.

Moreover, the present study determined that there was a decrease in the Na-K-ATPase activity *in vitro* and *in vivo* from moderate alcohol. Other studies have investigated the effect of ethanol upon the Na-K-ATPase activity in hepatocyte and renal cells at concentrations equivalent to a BAC of 0.08% or above and found that acute ethanol does decrease the activity of the Na-K-ATPase in these organs as well (Pascale, Daino et al. 1989, Rodrigo, Novoa et al. 1996). However, a previous study conducted with an extremely high dosage of ethanol (450 mM) in the jejunum of hamsters did not find a change in Na-K-ATPase activity (Dinda, Beck et al. 1975). This differs from our results. It is possible that the difference between this previous study and our results stems from the differences in species, the duration of ethanol exposure and concentration used. Moreover, studies conducted afterward in the jejunum of hamsters showed that acute dosages of ethanol decreased the activity of the Na-K-ATPase (Hoyumpa, Patwardhan et al. 1981), which does support our findings.

This decrease in Na-K-ATPase activity results in an altered sodium gradient in response to moderate ethanol both *in vitro* and *in vivo*. Thus, at least part of the inhibition of Na-glucose co-transport at the cellular level was secondary to this altered Na-extrusion capacity of the cell. The degree to which the Na-K-ATPase is responsible for the inhibition of glucose absorption can be further investigated. However, was the sodium-dependent glucose co-transporter SGLT1 directly affected? Thus, BBMV studies were undertaken. Again, moderate ethanol reduced Na-glucose co-transport in BBMVs prepared from ethanol-treated SD rats. Therefore, there are two mechanisms occurring in response to ethanol: there is a decrease in sodium-dependent glucose absorption through SGLT1 at the level of the co-transporter itself as well as at the level of the

sodium gradient produced by the Na-K-ATPase. Previous studies examining the effect of ethanol on glucose absorption in BBMVs have found that direct incubation of the BBMVs with 4% v/v ethanol reduced the absorption of glucose, as in our studies (al-Balool and Debnam 1989).

We determined that moderate ethanol does not affect the protein expression of the SGLT1 at the whole cell homogenate and BBM levels. The effect of ethanol on protein expression of SGLT1 has not been previously shown in the literature. Ethanol has been shown to increase the protein expression of the vitamin C co-transporters in the intestine in response to a 25% ethanol diet for two weeks in Kunming mice (Guo, Wang et al. 2013). On the other hand, thiamine transporter-1 protein and mRNA expression levels were decreased in response to two through six weeks of ethanol (Subramanya, Subramanian et al. 2010). Clearly, then, moderate ethanol's effect on SGLT1 is secondary to the altered affinity of the sodium-dependent glucose co-transporter as compared to diminished protein levels in the affected cells. Changes in phosphorylation and/or glycosylation may affect the affinity of SGLT1. Indeed, it has previously been shown that constitutive nitric oxide reduces SGLT1's affinity for glucose by altering the glycosylation of the protein (Arthur, Coon et al. 2014).

**Conclusions**

Many prior studies have determined the effect of large doses of ethanol on a variety of gastrointestinal functions. However, these studies have not investigated the effect of a moderate dose of ethanol. In this study, we used a moderate dose of ethanol equivalent to a BAC of 0.04%, which is achieved monthly by most of the population of the United States, making this dose very relevant (NIAAA). Clearly, this study demonstrates that even a modest dose of ethanol can have significant effects on important nutrient assimilation in the mammalian intestine. Moderate consumption of ethanol inhibits intestinal glucose assimilation. It specifically inhibits SGLT1.

The mechanism of inhibition is secondary to the reduced affinity of SGLT1 for glucose without a change in the number of co-transporters in enterocytes. These observations are similar both *in vitro* and *in vivo*. In conclusion, this novel study, using a moderate dose of ethanol, describes a possible mechanism for the malnutrition commonly seen in chronic alcoholics and thus has potential implications for the treatment of moderate ethanol-dependent malnutrition.

**Author Contributions**

Conceptualization, U.S.; Funding acquisition, U.S.; Investigation, M.B., and S.S.; Methodology, M.B., and S.S.; Writing, review and editing, M.B., S.S., J.H., S.A., and U.S.

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**Conflicts of Interest**

The authors have declared that no conflicts of interest exist.

# CHAPTER 6

In these studies, we were able to establish that moderate ethanol has a clear effect on the sodium-dependent glutamine and glucose, via B0AT1 and SGLT1, co-transport, respectively, in intestinal epithelial cells. However, we also showed that moderate ethanol affects a variety of other cell functions, which is in part explored in this chapter.

## FUNCTION OF THE NA-K-ATPASE IN REPONSE TO MODERATE ETHANOL

We determined in our previous studies that moderate ethanol significantly inhibited the activity of the Na-K-ATPase. This assay describes the activity of the Na-K-ATPase through inorganic phosphate release. Therefore, to also understand if the exchange of potassium ions, or function, of the Na-K-ATPase was also affected, we conducted rubidium uptakes in IEC-18 cells exposed to one and twelve hours of moderate ethanol. We determined that ethanol significantly decreased $^{86}$Rb uptake in ethanol-treated cells at both time points (Figure 26; 1070±10.1 pmol/mg protein•min in controls vs 603±122 in ethanol-treated cells at 1-hour, $p<0.05$, $n=3$).

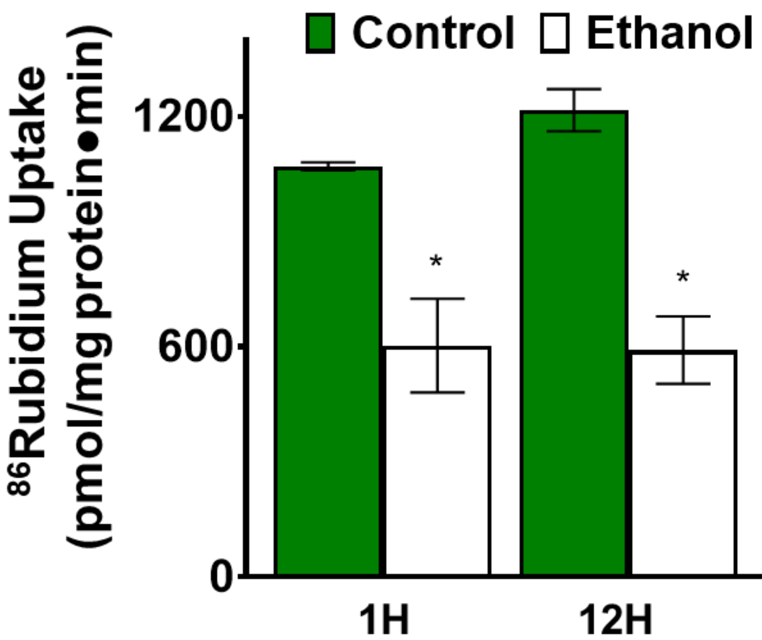


**Figure 26: One and twelve hours of moderate ethanol significantly reduced $^{86}$rubidium uptake in IEC-18 cells (*, $p<0.05$, n=3).** Error bars represent the SEM (1H: $p<0.05$; 12H: $p<0.01$).

Therefore, moderate ethanol affects the Na-K-ATPase in two ways: by a decrease in the release of inorganic phosphate as well as a decrease in the uptake of potassium ions, as measured with the rubidium uptakes. Further experiments were conducted examining the protein expression of the α1 subunit in response to moderate ethanol.

**Immunofluorescence intensity of the Na-K-ATPase in response to moderate ethanol**

This large drop in Na-K-ATPase activity could be due to many mechanisms. Previous studies have shown that the activity and expression of the Na-K-ATPase can be affected by ethanol (Hoyumpa, Patwardhan et al. 1981, Johnson and Crider 1989, Pascale, Daino et al. 1989, Rodrigo, Vergara et al. 1991, Rodrigo, Novoa et al. 1996, Rodrigo and Thielemann 1997, Otis, Mitchell et al. 2008, Rasic-Markovic, Krstic et al. 2008). Using ICC, ethanol significantly and slightly decreased the immunofluorescence intensity of the Na-K-ATPase α-1 subunit in IEC-18

cells (Figure 27, p<0.05, n=4). The methodology was the same as described in chapter four. Primary antibody raised in mouse (Sigma-Aldrich 05-369) and the appropriate anti-mouse AlexaFluor 488 secondary antibodies were used (ThermoFisher A28175).

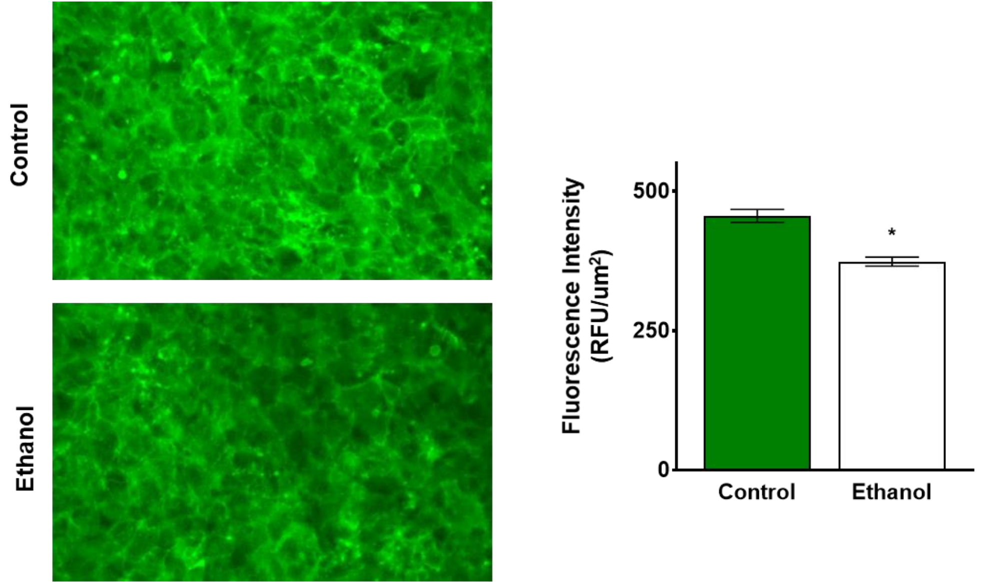


**Figure 27: Moderate ethanol decreased immunofluorescence intensity of the Na-K-ATPase α-1 subunit in IEC-18 cells (*, p<0.05, n=4).** Each sample is an average of at least 5 different images obtained using a 20X objective. Error bars represent the SEM.

Further studies should be conducted to fully understand the effect of moderate ethanol on the protein expression of the Na-K-ATPase.

**Ethanol's effect on ZO-1 expression**

In order to determine alternative possible mechanisms of ethanol's action on intestinal epithelial cells, the tight junction protein ZO-1 was examined using ICC in IEC-18 cells exposed to one and twelve hours of moderate ethanol. As ethanol has been shown to affect tight junctions, it was important to determine if there were any changes in ZO-1 expression (Cresci, Bush et al. 2014, Elamin, Masclee et al. 2014). Moderate ethanol did not change the expression of ZO-1

(Figure 28; p>0.05, n=4). The methodology used was described in chapter four. The primary antibody was raised in mouse (ThermoFisher 33-9100) and the appropriate anti-mouse AlexaFluor 488 secondary antibodies were used (ThermoFisher A28175).

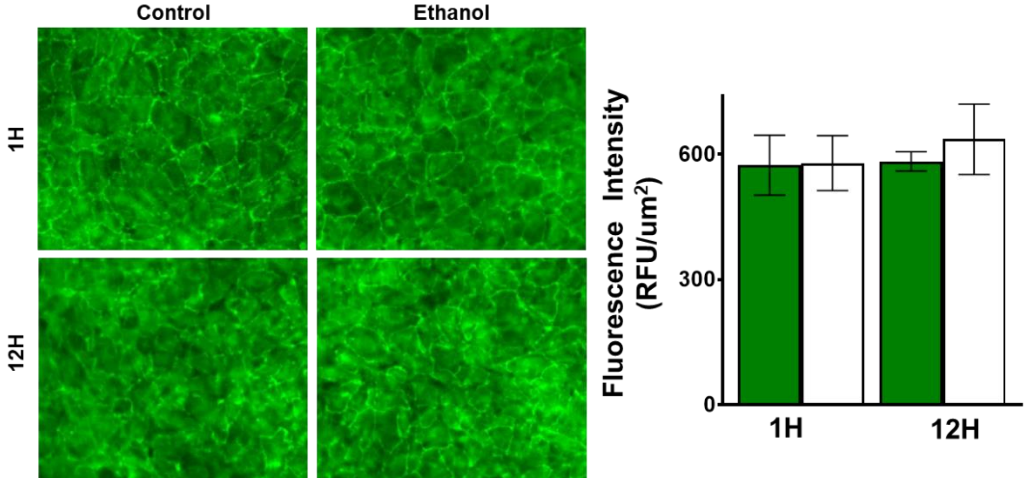


**Figure 28: Ethanol did not alter ZO-1 immunofluorescence intensity in IEC-18 cells (n=4).** Each sample is an average of at least 5 different images obtained using a 20X objective. Error bars represent the SEM (1H: p>0.05; 12H: p>0.05).

Clearly, moderate ethanol does not significantly affect the tight junction protein ZO-1. Furthermore, the passive movement of glutamine and glucose were measured in all uptake experiments. No significant differences existed in the passive movement of glutamine and glucose between control and ethanol-treated cells at one hour (Figure 29A; 366±44.1 pmol/mg protein•min in controls vs 383±43.3 in ethanol-treated cells for glutamine uptake, p>0.05, n=6) (Figure 29B; 1119±175 pmol/mg protein•min in controls vs 1086±146 in ethanol-treated cells for glucose uptake, p>0.05, n=6). Therefore, moderate ethanol does not have a significant effect on the passive movement of nutrients in IEC-18 cells.

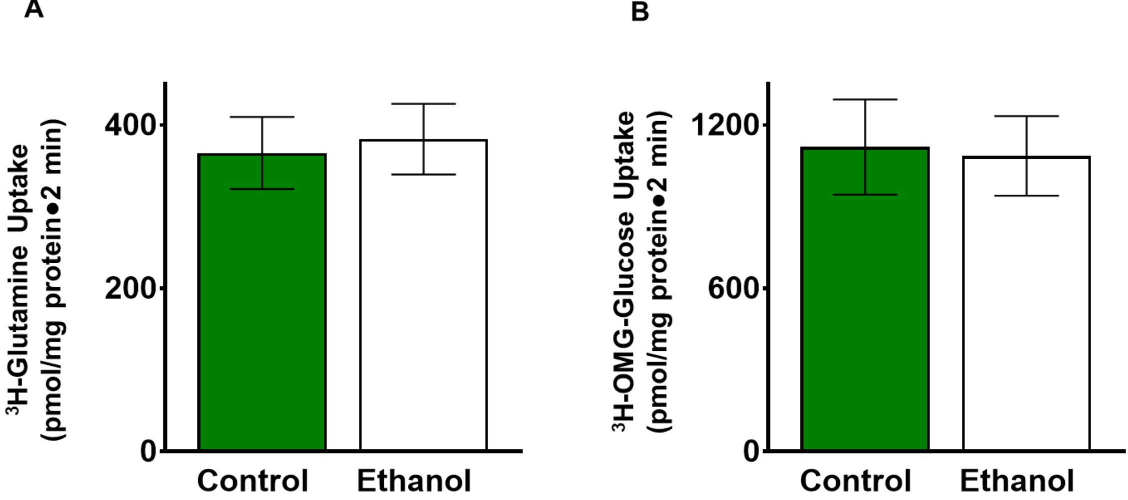


**Figure 29: Moderate ethanol does not change passive nutrient uptake in IEC-18 cells. A)** Passive glutamine uptake in response to moderate ethanol for 1-hour ($p>0.05$, n=6). **B)** Passive glucose uptake in response to moderate ethanol for 1-hour (n=6). Error bars represent the SEM.

**Conclusions**

Overall, this dissertation has clearly shown that moderate ethanol decreases sodium-dependent glutamine and glucose co-transport through B0AT1 and SGLT1, respectively, in intestinal epithelial cells along the small intestine both *in vitro* and *in vivo.* Furthermore, the work examined in this dissertation has revealed a novel and broad field of investigation for future studies in other nutrient transporters in response to moderate ethanol along the small intestine.

# CHAPTER 7

# DISCUSSION

The small intestine is responsible for absorbing all the essential dietary nutrients the mammalian body requires to efficiently function. The small intestine interacts with a wide variety of ingested substances, including alcohol. Alcohol interacts with the small intestine in almost 90% of the population of the United States at some point (NIAAA). However, the interaction between alcohol and the absorption of nutrients along the small intestine at the level of the nutrient co-transporter has not been fully investigated. More specifically, further research is required to understand how moderate ethanol affects the small intestine's two primary fuel sources: glutamine and glucose. Therefore, this dissertation focused on the effect of moderate ethanol on the sodium-dependent glutamine and glucose co-transporters B0AT1 and SGLT1, respectively, in the small intestine.

## Novelty and rationale

This dissertation is striking, due to the inherent novelty of the subject matter. For over fifty years, the relationship between ethanol and nutrition has been heavily scrutinized. However, because so much of the research focusing on ethanol's effect on nutrient absorption was conducted in the late 1900s, the advent of new technologies and discoveries of novel nutrient co-transporters has opened this field to new avenues of research, including the effect of ethanol on the sodium-dependent nutrient co-transporters B0AT1 and SGLT1. This new connection between ethanol and nutrient co-transporters is fascinating because it demonstrates ethanol's influence at a subcellular level.

### The novelty of ethanol and glutamine research

The association between alcohol use and glutamine absorption has never been investigated in the mammalian system. As most ethanol and nutrition research occurred in the late 1900s, this lack of research could be due to the delayed onset of glutamine-associated research. The importance of glutamine in the intestinal epithelium has only been emphasized since the early 2000s. In 2002, glutamine deprivation in rat pups displayed villus blunting in the ileum and decreased epithelial junctions (Potsic, Holliday et al. 2002). As of 2015, researchers were still clarifying this relationship. Researchers showed that glutamine deprivation reduced porcine intestinal epithelial cell numbers within eight hours (Zhu, Lin et al. 2015). Clearly, glutamine is an essential nutrient in the small intestine, but this connection was not discovered until the past twenty years. As a result, the effect of ethanol on glutamine absorption has not yet been investigated.

### The novelty of ethanol and glucose research

On the other hand, the association between decreased glucose absorption and alcohol use has been well investigated since the 1980s (Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981, Cobb, Van Thiel et al. 1983, al-Balool and Debnam 1989, Money, Petroianu et al. 1990, Kaur, Kaur et al. 1995, Bode and Bode 2003, Yunus, Awad et al. 2011). However, the SGLT1 co-transporter was not characterized until 1987 (Hediger, Coady et al. 1987) Thus, the relationship between moderate ethanol and the specific sodium-dependent SGLT1 co-transporter in the small intestine has not been investigated until now.

### The use of moderate ethanol

The dosage of ethanol was highly considered before conducting this dissertation. Commonly, research studies use doses of ethanol that can be rarely achieved by an average adult

(greater than a BAC of 0.2%). Levels of lethal BAC were measured in 175 adults and was averaged to be 0.355% (Heatley and Crane 1990). However, despite this being the lethal limit of alcohol consumption, some studies have used this high concentration of alcohol (Hoyumpa, Breen et al. 1975, Dinda and Beck 1977, Lemos, Azevedo et al. 2005, Romanoff, Ross et al. 2007, Li, Xu et al. 2008). Other studies focus on more relatable doses, achieved by chronic alcoholics (Hoyumpa, Breen et al. 1975, Hajjar, Tomicic et al. 1981, Hoyumpa, Patwardhan et al. 1981, Antonson and Vanderhoof 1983, Pascale, Daino et al. 1989, Rodrigo, Vergara et al. 1991, Kaur, Kaur et al. 1995, Rodrigo, Novoa et al. 1996, Rodrigo and Thielemann 1997, Otis, Mitchell et al. 2008, Joshi, Mehta et al. 2009, Sabino, Petroianu et al. 2010, Subramanya, Subramanian et al. 2010, Nogales, Ojeda et al. 2011, Guo, Wang et al. 2013, Subramanian, Subramanya et al. 2013). Few studies use doses lower than that seen in chronic alcoholics (Krishnamra and Limlomwongse 1987, Hamid, Wani et al. 2007). Therefore, we chose to use a dosage of ethanol that is achieved not only by chronic alcoholics, but by moderate drinkers: a BAC of 0.04%. This dosage of moderate ethanol is achieved by over half of the population of the United States per month. Therefore, our dosage used was considered very translational to the human condition (NIAAA, Gunzerath, Faden et al. 2004).

**Ethanol, B0AT1 and SGLT1**

The absence of research conducted directly on the B0AT1 and SGLT1 co-transporters in the intestinal ileum using a moderate dosage of ethanol allows this dissertation to stand alone in the field of alcohol and nutrition research.

**Aim**

The aim of this study was to investigate the effect of moderate ethanol on the sodium-dependent glutamine and glucose co-transporters in the small intestine: B0AT1 and SGLT1,

respectively. We demonstrated these findings in the ileum of the small intestine, due to its compensatory mechanisms of nutrient absorption. If ethanol affects nutrient absorption at the ileum, it is likely that transport is affected throughout the intestine (Koopmans 1990). In order to accomplish this goal, we began by testing the effect of ethanol on nutrient absorption in a rat intestinal epithelial cell line (IEC-18).

## ***In vitro* experimentation**

### Preliminary data

#### Dosage

The initial steps of this dissertation involved establishing the dose of ethanol to use. Even sub-moderate doses of ethanol (0.02% BAC) significantly decreased the absorption of glutamine in IEC-18 cells. This is the dosage equivalent of less than a standard alcoholic beverage for women and one alcoholic beverage for men. Few studies have seen a physiological effect on nutrient co-transport at this small amount of ethanol with one of the few being conducted by Krishnamra and colleagues (Krishnamra and Limlomwongse 1987, Hamid, Wani et al. 2007). Most studies are conducted with chronic doses of ethanol (Dinda, Beck et al. 1975, Dinda and Beck 1977, Beck and Dinda 1981, Dinda and Beck 1981, Hajjar, Tomicic et al. 1981, al-Balool and Debnam 1989, Kaur, Kaur et al. 1995, Li, Xu et al. 2008, Sabino, Petroianu et al. 2010, Subramanya, Subramanian et al. 2010, Guo, Wang et al. 2013, Subramanian, Subramanya et al. 2013). In all, the dosage equivalent of a 0.04% BAC was selected due to its translational applicability in humans, as it is the equivalent of a moderate dosage of ethanol.

#### Cell viability

The next logical step in our investigation of ethanol on nutrient absorption was to assess if this dosage of ethanol could harm intestinal epithelial cells. A study conducted in neuronal-like

pheochromocytoma (PC12) cells, using the same dosage of ethanol used in our studies (0.04% BAC), showed that ethanol caused cell death after consistent exposure for 24 hours using trypan blue assays (Luo, West et al. 1999). Using trypan blue and LDH assays, we determined that there was no change in cell viability in response to this dosage of ethanol over one to twelve hours in IEC-18 cells, similar to previous studies (Luo, West et al. 1999).

**BLM versus BBM treatment**

As ethanol has been shown to interact with the ileum of the small intestine along the BLM of the cell, through the vasculature, the effect of ethanol on the BLM was conducted using transwell studies. The effect of moderate ethanol on glutamine uptake was the same whether treated on the BLM side or the BBM. Therefore, studies were conducted using apical treatment to reduce significant time and fiscal cost.

**Acetaldehyde**

We also assessed the action of ethanol's primary toxic metabolite, acetaldehyde, as a possible mechanism of this decrease in nutrient absorption. One study has shown that acetaldehyde can mediate decreased folate absorption in the colon of chronic ethanol-administered rats. The decreased folate absorption was reversed with the administration of 50 mg/kg ciprofloxacin, which decreased the proliferation of bacteria in the colon, and therefore the inherent acetaldehyde production (Homann, Tillonen et al. 2000). Therefore, it is important to always consider the effect of the primary metabolite of ethanol metabolism and its potential effects on nutrient absorption.

We measured 11.4 µM acetaldehyde after one hour of moderate ethanol exposure in IEC-18 cells. Other studies have found comparable amounts of acetaldehyde in the small intestine. One study using wildtype mice found plasma ethanol levels equivalent to approximately 0.6 g/dL

ethanol led to approximately 15 μM excess acetaldehyde in ethanol-treated mice. This directly supports the acetaldehyde concentrations used in our studies (Chaudhry, Samak et al. 2015). Further studies in the upper small intestine of Wistar rats show that concentrations of acetaldehyde can reach approximately 7.9 μM, but in response to 34 mM ethanol. This is significantly higher than the concentration used in our studies, but the values may vary due to the influence of the liver in this study compared to our *in vitro* experiment as well as intestinal segment differences (Chaudhry, Samak et al. 2015).

Therefore, we administered 15 μM acetaldehyde to IEC-18 cells for one hour and found that both glutamine and glucose absorption was unaffected by this dosage of acetaldehyde. Thus, ethanol, not its primary toxic metabolite acetaldehyde, is causing the decrease in nutrient absorption in these cells.

**Ethanol's effect on glutamine absorption *in vitro***

As a main finding of this dissertation, we determined that moderate ethanol significantly decreased sodium-dependent glutamine absorption in IEC-18 cells over one to twelve hours. No other study has shown that ethanol significantly decreases sodium-dependent glutamine absorption in the small intestine. As ethanol decreases the absorption of a variety of nutrients along the small intestine, it was not surprising that glutamine absorption was decreased in response to ethanol in our studies (Chang, Lewis et al. 1967, Dinda, Beck et al. 1975, Hoyumpa, Breen et al. 1975, Dinda and Beck 1977, Racusen and Krawitt 1977, Hoyumpa 1980, Dinda and Beck 1981, Hoyumpa, Patwardhan et al. 1981, Antonson and Vanderhoof 1983, Cobb, Van Thiel et al. 1983, Middleton 1986, Krishnamra and Limlomwongse 1987, al-Balool and Debnam 1989, Money, Petroianu et al. 1990, Pfeiffer, Schmidt et al. 1992, Kaur, Kaur et al. 1995, Bode and Bode 2003, Lemos, Azevedo et al. 2005, Hamid, Wani et al. 2007, Romanoff, Ross et al. 2007,

Li, Xu et al. 2008, Subramanya, Subramanian et al. 2010, Yunus, Awad et al. 2011, Guo, Wang et al. 2013, Subramanian, Subramanya et al. 2013, Thakur, Rahat et al. 2015). However, few studies have investigated ethanol's effect on the individual nutrient co-transporters along the small intestine, with none in an ileal cell line (Hamid, Wani et al. 2007, Li, Xu et al. 2008, Joshi, Mehta et al. 2009, Subramanya, Subramanian et al. 2010, Nogales, Ojeda et al. 2011, Guo, Wang et al. 2013, Subramanian, Subramanya et al. 2013, Thakur, Rahat et al. 2015). We determined that ethanol decreased the maximal velocity of the sodium-dependent B0AT1 co-transporter ($V_{max}$) without a change in the affinity of the co-transporter ($K_m$), suggesting that the number of B0AT1 co-transporters was decreased. We determined that moderate ethanol did decrease B0AT1 protein expression at the whole cell homogenate and BBM, explaining this decrease in $V_{max}$ values. Few other studies have demonstrated a change in the kinetic parameters of a co-transporter in response to ethanol (Kaur, Kaur et al. 1995, Hamid, Wani et al. 2007, Nogales, Ojeda et al. 2011). Previous studies have shown that B0AT1 is decreased in response to chronic intestinal inflammation in the rabbit ileum (Saha, Arthur et al. 2012). Chronic intestinal inflammation also decreased the $V_{max}$ values for B0AT1, without a change in affinity ($K_m$). Overall, in response to intestinal stressors such as moderate ethanol and chronic intestinal inflammation, sodium-dependent glutamine co-transport is decreased.

Furthermore, changes of protein co-transporter levels in response to ethanol have been shown before as well, but never for B0AT1 (Joshi, Mehta et al. 2009, Subramanya, Subramanian et al. 2010, Guo, Wang et al. 2013, Subramanian, Subramanya et al. 2013, Thakur, Rahat et al. 2015). Overall, moderate ethanol decreased B0AT1 protein expression at the BBM, leading to decreased glutamine absorption in IEC-18 cells.

**Ethanol's effect on glucose absorption *in vitro***

At this point, it was important to consider if moderate ethanol could affect other sodium-dependent nutrient co-transporters. Therefore, another sodium-dependent nutrient co-transporter was tested: SGLT1. Previously, there was a lot of background information on ethanol and glucose absorption (Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981, Cobb, Van Thiel et al. 1983, al-Balool and Debnam 1989, Money, Petroianu et al. 1990, Kaur, Kaur et al. 1995, Yunus, Awad et al. 2011), but no other studies have been conducted using moderate ethanol in the ileum of the small intestine. We determined that sodium-dependent glucose co-transport was also decreased in response to ethanol, as described in the previous studies (Dinda, Beck et al. 1975, Dinda and Beck 1977, Dinda and Beck 1981, Cobb, Van Thiel et al. 1983, al-Balool and Debnam 1989, Money, Petroianu et al. 1990, Kaur, Kaur et al. 1995, Yunus, Awad et al. 2011). Furthermore, kinetic studies showed increased $K_m$ values, suggesting a decrease in the affinity of the glucose co-transporter to its substrate. There was no change in $V_{max}$ values, which was subsequently supported with no changes to SGLT1 protein expression in the whole cell homogenate and BBM, as seen in Western blots. Previous studies have shown decreases in the affinity of nutrient co-transporters in response to ethanol as well (Hamid, Wani et al. 2007, Nogales, Ojeda et al. 2011). Another study investigating glucose absorption found no change in $K_m$ values, but a decrease in $V_{max}$ values in response to ethanol. The difference between this study and our results could be due to the difference in ethanol concentration used between these studies (20% v/v ethanol for 40 days in rats) (Kaur, Kaur et al. 1995). No other study has reported changes to SGLT1 protein expression in response to any dose of ethanol. Overall, we demonstrated that ethanol does not only inhibit sodium-dependent B0AT1 co-transport in the small intestine, but also sodium-dependent SGLT1 co-transport as well.

**Ethanol's effect on Na/H exchange *in vitro***

This change in SGLT1-mediated glucose absorption at the level of the co-transporter was fascinating. But it brought up a very important question: is this a system-wide inhibition of all sodium absorptive processes in the intestinal BBM? This hypothesis was fathomable due to ethanol's inhibitory effect on the sodium-gradient (Hoyumpa, Patwardhan et al. 1981, Rodrigo, Vergara et al. 1991, Rodrigo, Novoa et al. 1996, Rodrigo and Thielemann 1997). The primary sodium absorptive pathways in intestinal epithelial cells is through SGLT1 and Na/H exchange. Since SGLT1 has been shown to be coupled to Na/H exchange through intracellular nitric oxide (Coon, Kekuda et al. 2008), Na/H exchange was conducted in IEC-18 cells exposed to moderate ethanol for one hour. No change was demonstrated in Na/H exchange in response to moderate ethanol which demonstrates that the effect of ethanol on nutrient co-transport is not a system-wide depressant of the sodium absorption processes in the small intestine. Considering that SGLT1 and the Na/H exchanger NHE3 have previously been shown to be coupled, regulated by constitutive nitric oxide in rabbit intestinal epithelial cells (Coon, Kekuda et al. 2008), it was surprising to see that Na/H exchange was also not affected by moderate ethanol. However, recent studies have demonstrated that Na/H exchange is not always affected in conjunction with SGLT1 (Manoharan, Sundaram et al. 2018). Researchers demonstrated a decrease in SGLT1 activity in response to chronic intestinal inflammation, but not in Na/H exchange. Therefore, it is reasonable to accept that SGLT1 can be affected by ethanol, without a change in Na/H exchange (Manoharan, Sundaram et al. 2018).

***In vivo* experimentation**

Ethanol use, even at a moderate dose, has whole body effects due to its transit through the vasculature (Bode and Bode 2003, Krenz and Korthuis 2012). In order to provide a more

convincing translational study, involving the enteric, vascular, and muscular systems, an *in vivo* model was used. Despite these added variables, the inhibitory effect of moderate ethanol on sodium-dependent glucose co-transport was still observed in Sprague Dawley rats administered an intragastric gavage of 2 g/kg ethanol (equivalent to a BAC of 0.04%). Na-K-ATPase activity and glucose absorption were both suppressed *in vivo* as in the *in vitro* model. Furthermore, similar results were demonstrated in kinetic studies, Western blots, and immunohistochemistry *in vivo* as *in vitro*. These similar results *in vivo* and *in vitro* only help to demonstrate the translational applicability of the effect of ethanol on nutrient absorption in the small intestine.

Furthermore, in the *in vivo* model, BBMV uptake experiments were able to be prepared from control and ethanol-treated rats. BBMVs separate the BLM-located Na-K-ATPase from the apical BBM. Resulting nutrient uptakes showed that glucose absorption was still inhibited in response to moderate ethanol without the influence of the Na-K-ATPase. Overall, this demonstrates that ethanol specifically affects the nutrient co-transporter itself, in addition to its effect on the sodium gradient. BBMVs were also used in studies examining the effect of ethanol on glucose absorption in the jejunum of hamsters and Sprague Dawley rats (Dinda and Beck 1981, al-Balool and Debnam 1989). However, these studies administered the respective dosages of ethanol directly onto the vesicles, whereas our studies did not. Overall, all of these studies using BBMV uptakes found that ethanol directly inhibits glucose absorption at the co-transporter itself without the influence of the Na-K-ATPase.

## Additional experiments

### The effect of ethanol on the Na-K-ATPase

The effect of ethanol on the BLM Na-K-ATPase needed to be investigated if we were to completely understand the effect of ethanol on the BBM-located nutrient co-transporters. We

found that the decrease in Na-K-ATPase activity was inhibited by more than half of its normal activity levels. This was an immense inhibition. However, similar inhibitions of Na-K-ATPase activity have been demonstrated in previous studies. For example, binding of the Na-K-ATPase regulatory protein phospholemman reduces Na-K-ATPase activity by approximately 40% (Mishra, Habeck et al. 2015). Phosphorylation of this protein by PKC can affect the function of this pump, but only the α-2 isoform in *Xenopus llaevis* oocytes (Bibert, Roy et al. 2008). Moreover, the Na-K-ATPase can experience approximately a 60% drop at 20 mmol/L sodium when exposed to 2 mmol/L oxidized glutathione in the human skeletal muscle (Juel, Hostrup et al. 2015). In all, the large drop in Na-K-ATPase activity has been shown multiple times throughout the literature (Bibert, Roy et al. 2008, Juel, Hostrup et al. 2015, Mishra, Habeck et al. 2015).

We demonstrated similar inhibition of Na-K-ATPase activity *in vivo* in response to moderate ethanol. The assay used to analyze Na-K-ATPase activity was via inorganic phosphate release. This pump requires the hydrolysis of ATP to function, making inorganic phosphate release a very reasonable measurement of Na-K-ATPase activity. However, the Na-K-ATPase also maintains important concentration gradients in cells by exchanging three sodium ions out of the cell for two potassium ions into the cell. Moderate ethanol may also be affecting this important function of the Na-K-ATPase. Therefore, we measured the function of the Na-K-ATPase using rubidium uptakes, which approximately measures how much potassium the Na-K-ATPase is transporting into the cell. We established that moderate ethanol significantly decreased uptake of rubidium in IEC-18 cells. Therefore, moderate ethanol also significantly inhibits the functional activity of the Na-K-ATPase in IEC-18 cells.

However, how is the function and activity of the Na-K-ATPase affected by moderate ethanol? We found that there was a significant decrease in the immunofluorescence intensity of the Na-K-ATPase α-1 subunit in IEC-18 cells exposed to moderate ethanol for one hour. This decrease in immunofluorescent intensity could explain the decrease in activity. The Na-K-ATPase has been previously shown to be altered in intestinal epithelial cells. In intestinal epithelial cells of chronically inflamed rabbits, the activity of the Na-K-ATPase was decreased, but was not due to changes in protein expression or relative mRNA abundance of the α-1 and β-1 subunits (Saha, Manoharan et al. 2015). It was suggested that ankyrin, part of the protein cytoskeleton necessary for the correct trafficking of the Na-K-ATPase, was affected by chronic intestinal inflammation. Ankyrin protein expression was significantly diminished in intestinal epithelial cells, which resulted in improper trafficking to the BLM, and the decrease in Na-K-ATPase activity (Saha, Manoharan et al. 2015). Clearly, the Na-K-ATPase can be affected by improper trafficking. Moreover, activation of the Na-K-ATPase-associated Src kinase has been shown to induce endocytosis of the Na-K-ATPase (Xie and Xie 2005). Further research is necessary to fully understand the effect of ethanol on the Na-K-ATPase.

**Effect of moderate ethanol on ZO-1 expression**

Possible mechanisms of this decrease in nutrient absorption could be due to the integrity of the tight junctions in these intestinal epithelial cells. Fortunately, the glutamine and glucose uptake studies conducted in intestinal epithelial cells account for the passive movement of glutamine and glucose into the intestinal epithelial cells. There was no significant difference in the passive movement of these nutrients in response to moderate ethanol. However, it has been demonstrated that intestinal epithelial cells can have alterations to their transmembrane proteins, like tight junctions, in response to ethanol (Cresci, Bush et al. 2014, Elamin, Masclee et al.

2014). Therefore, in IEC-18 cells, ZO-1 immunofluorescence intensity was examined. ZO-1 immunofluorescence intensity was not significantly changed in response to one and twelve hours of moderate ethanol. Although this does not fully describe the localization of this tight junction protein, as the protein could be translocated intracellularly, future studies examining this phenomenon can elucidate if this dosage of moderate ethanol removes tight junction proteins like ZO-1 from the cellular junctions and studies measuring transepithelial resistance can ensure the stability of the intestinal tight junctions.

**Possible mechanisms**

**Ethanol-mediated decrease in glutamine absorption *in vitro***

It is quite clear that moderate ethanol decreases glutamine absorption at the level of the sodium-dependent glutamine co-transporter in response to a moderate dosage of ethanol. We demonstrated in IEC-18 cells that moderate ethanol decreased the protein expression of B0AT1 in whole cell homogenates and BBM, concurrently with a visible decrease in immunofluorescence intensity. Clearly, ethanol is causing a removal of B0AT1 protein expression from the BBM, which is supported by our kinetic studies demonstrating a decrease in $V_{max}$ values. Previous studies in our lab have demonstrated that B0AT1 is also inhibited through a reduction in BBM co-transporters during states of chronic intestinal inflammation (Saha, Arthur et al. 2012). Researchers demonstrated that inducible nitric oxide, which is elevated during chronic intestinal inflammation, regulates the inhibition of B0AT1 at the BBM of rabbit intestinal villus cells. This was demonstrated through use of an inducible nitric oxide inhibitor, L-NIL, which reversed the inhibition of B0AT1 (Arthur and Sundaram 2015). This reversal was also shown through administration of the glucocorticoids (Arthur, Saha et al. 2012) via prostaglandins in the cyclooxygenase pathways (Arthur, Singh et al. 2018). Overall, B0AT1 has

also been shown to be removed from the BBM under states of chronic intestinal inflammation, due to immune system modulators, which may be similar to what is occurring in our study regarding ethanol. Ethanol could be causing signaling cascades that could lead to the removal of B0AT1 from the BBM.

One of these signaling cascades could be initiated by PKC. In PKC siRNA studies conducted in IEC-18 cells, the inhibitory effect on glutamine absorption was reversed. Although this is only one of many possible mechanisms, clearly PKC has an effect on glutamine absorption in response to moderate ethanol. Further studies are needed to describe this mechanism.

**Ethanol-mediated decrease in glucose absorption *in vitro* and *in vivo***

Ethanol also causes a decrease in sodium-dependent glucose co-transport *in vitro* and *in vivo*; however, moderate ethanol differentially regulates B0AT1 and SGLT1. The mechanism of moderate ethanol on SGLT1 is through increased *Km* values, suggesting a decrease in the affinity of the co-transporter to its substrate.

The change in affinity of SGLT1 in response to moderate ethanol could be caused by post-translational modifications of SGLT1. SGLT1 has been shown to be phosphorylated or glycosylated in response to cell mediators (Arthur, Coon et al. 2014). In previous studies, inhibition of constitutive nitric oxide has shown an inhibition of SGLT1 through a reduction in the affinity in IEC-18 cells, which was shown to be due to the glycosylation of SGLT1 (Arthur, Coon et al. 2014). Ethanol could be initiating signaling mechanisms which could activate post-translational modifications of SGLT1, causing this decrease in SGLT1-mediated glucose absorption in response to moderate ethanol. Further research is necessary to explore this hypothesis.

## FUTURE DIRECTIONS

The signaling mechanisms causing these differing effects on glutamine and glucose absorption in intestinal epithelial cells can be elucidated in the future. Previous studies have found that B0AT1 and SGLT1 can be affected by different signaling mechanisms (Arthur, Coon et al. 2014, Arthur, Singh et al. 2018). Furthermore, the mechanism of action of ethanol on the Na-K-ATPase in intestinal epithelial cells has yet to be clarified. Future studies can focus on determining ethanol's effect on the necessary sodium-gradient for sodium-dependent co-transporters.

Ethanol influences nutrient absorption along the small intestine (Chang, Lewis et al. 1967, Dinda, Beck et al. 1975, Hoyumpa, Breen et al. 1975, Dinda and Beck 1977, Racusen and Krawitt 1977, Hoyumpa 1980, Dinda and Beck 1981, Hoyumpa, Patwardhan et al. 1981, Antonson and Vanderhoof 1983, Cobb, Van Thiel et al. 1983, Middleton 1986, Krishnamra and Limlomwongse 1987, al-Balool and Debnam 1989, Money, Petroianu et al. 1990, Pfeiffer, Schmidt et al. 1992, Kaur, Kaur et al. 1995, Bode and Bode 2003, Lemos, Azevedo et al. 2005, Hamid, Wani et al. 2007, Romanoff, Ross et al. 2007, Li, Xu et al. 2008, Subramanya, Subramanian et al. 2010, Yunus, Awad et al. 2011, Guo, Wang et al. 2013, Subramanian, Subramanya et al. 2013, Thakur, Rahat et al. 2015). Chronic use of alcohol can lead to an alcohol-dependent malnutrition that may be able to be modulated by supplementation of specific nutrients. Supplementation with glutamine may be beneficial in cases of intestinal disruption linked with heavy alcohol use, which may help prevent the dangerous translocation of endotoxins seen in chronic alcoholics causing liver damage. This is a prime potential avenue for future ethanol and glutamine research.

Moreover, the ethanol-mediated phenomenon described in this dissertation also provides a potential mechanism of onset for alcohol-dependent malnutrition. Future studies should investigate potential mediators that may ameliorate this condition. Furthermore, future investigations can explore the involvement of ethanol-dependent inhibition of glucose absorption in order to aid in glucose-associated diseases like diabetes and obesity. Overall, there are many potential avenues for future research in the field of ethanol and sodium-dependent nutrient co-transport.

## CONCLUSIONS

We demonstrated that a moderate dosage of ethanol significantly decreased sodium-dependent glutamine and glucose co-transport in a rat intestinal epithelial cell line (IEC-18) and that moderate ethanol decreased sodium-dependent glucose co-transport in Sprague Dawley rats. We isolated the effect of ethanol into two separate mechanisms: at the level of the sodium-dependent nutrient co-transporters, B0AT1 and SGLT1, as well as to the sodium gradient set by the Na-K-ATPase. Furthermore, we showed that silencing PKC in IEC-18 cells reverses the effect of ethanol on sodium-dependent glutamine absorption which offers the first part of a probable mechanism for ethanol's effect on sodium-dependent glutamine absorption. Overall, this novel dissertation defined moderate ethanol's effect on the sodium-dependent nutrient co-transport in the small intestine. This dissertation and future experiments can help describe the onset of alcohol-dependent malnutrition.

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# APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

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Office of Research Integrity

April 11, 2019

Molly Butts
1425 Stewart Ave
Huntington, WV 25701

Dear Ms. Butts:

This letter is in response to the submitted dissertation abstract entitled *"The Effect of Moderate Alcohol Consumption on Sodium-dependent Nutrient Cotransport in Intestinal Epithelial Cells in vitro and in vivo."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #688. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity

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## APPENDIX B: ABBREVIATIONS

ADH – Alcohol dehydrogenase

ALDH – Aldehyde dehydrogenase

ATP – Adenosine triphosphate

BAC – Blood alcohol content

BBM – Brush border membrane

BBMV – Brush border membrane vesicle

BLM – Basolateral membrane

BSA – Bovine serum albumin

COX – Cyclooxygenase

cNOS – Constitutive nitric oxide synthase

CYP2E1 – Cytochrome P450 2E1

DMEM – Dulbecco's modified Eagle's medium

GI – Gastrointestinal

ICC – Immunocytochemistry

IEC – Intestinal epithelial cell

IHC - Immunohistochemistry

$P_i$ – Inorganic phosphate

iNOS – Inducible nitric oxide synthase

HIF – Hypoxia inducible factor

HNF – Hepatocyte nuclear factor

HSP – Heat shock protein

NAD – Nicotinamide adenine dinucleotide

NHE3 – N/H exchanger 3

NO – Nitric oxide

OMG – *O*-methyl-*D*-glucose

PBS – Phosphate buffered saline

PKC – Protein kinase C

PTP – Protein tyrosine phosphatase

ROS – Reactive oxygen species

SD – Sprague Dawley

SEM – Standard error of the mean

SLC – Solute carrier

TJ – Tight junction

TNF – Tumor necrosis factor

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*Email address: butts15@live.marshall.edu*

*Phone number: 716-679-8247*

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**Terese Winslow LLC, Medical Illustration**
714 South Fairfax Street, Alexandria, Virginia 22314
(703) 836-9121
terese@teresewinslow.com

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**APPENDIX D: CURRICULUM VITAE**

# Molly Rae Butts

1425 Stewart Avenue Huntington, WV 25701
716-679-8247 - butts15@live.marshall.edu

**Education**

Biomedical Research Ph.D. Candidate — July 2014 - Present
*Marshall University Huntington, WV*

*B.S. Allegheny College, Meadville, PA* — August 2010 - May 2014
Major in Neuroscience; minor in Spanish

**Publications**

Butts M.R., Singh S., Sundaram U. (2018). "The Novel Regulation of Ethanol on Sodium Dependent Glucose Co-Transport in Intestinal Epithelial Cells". Alcoholism: Clinical & Experimental Research.**42**: S1. 157A. Abstract.

Butts M.R., Singh S., Sundaram U. (2018). "Alcohol Uniquely Regulates Sodium-Glutamine Co-Transport in Intestinal Epithelial Cells". Gastroenterology. **154**: 6. S655-S656. Abstract.

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Singh S., Nepal N., Butts M., Parkulo T., Arthur S., Sundaram U. (2016). "Mammalian Intestinal Crypt to Villus Maturation Mediated Differentiation Na Glutamine Co-Transporters SN2 to B0AT1". Gastroenterology. **150:**4. S199. Abstract.

**Senior Thesis**

The Effect of Ethanol on BK Channel Current and Channel Density — May 2013 - May 2014
*Allegheny College, Meadville, PA*

- Designed, conducted and orally defended a unique independent research project under Dr. Lauren French
- Investigated the effect of ethanol exposure upon *Xenopus laevis* oocytes injected with BK channel RNA using electrophysiology
- Fifteen minutes of 50 mM ethanol exposure increased channel current by 70 percent
- Initiation of immunofluorescent experiments were conducted by expressing GFP tagged

BK channels in order to investigate channel trafficking

**Research Experience**

*Research Assistant to Dr. Uma Sundaram* April 2014 - Present
*Marshall University Biomedical Research Program, Huntington, WV*

- Established intestinal stem cell isolation and culture from mouse, rabbit and human samples
- Conducted general laboratory procedures including Western blots, protein assays, RT-QPCR, immunocytochemistry, immunohistochemistry, cell viability assays, radioactive whole cell uptakes, radioactive brush border membrane uptakes and radioactive kinetic studies
- Extensively worked with various mammalian vertebrate models including B6 and Tally-Ho mice, lean and obese Zucker rats, Sprague Dawley rats, and New Zealand White rabbits as well as with cell culture lines such as human epithelial colorectal adenocarcinoma cells (Caco2), intestinal epithelial cells (IEC-18), human embryonic kidney super topflash assay cells (HEK293-STF), R-spondin2 and Wnt3A producing cells lines
- Specialized in unique laboratory procedures including intestinal brush border membrane isolation, plasma membrane isolation, intestinal crypt and villus isolation in mice, rats and rabbits, oral gavage in rats and rabbits, reactive oxygen species assays, acetaldehyde assays and Na/K-ATPase assays

*Research Rotations* August 2014 - May 2015
*Marshall University Biomedical Research Program, Huntington, WV*

- Conducted four different laboratory rotations averaging 120 hours each
- Gained experience in general laboratory procedures including protein assays, Western blot techniques, cell culture, molecular biology procedures, mammalian animal experimentation, ELISA, hippocampal slice isolation, field potential recording and radioactive uptake experimentation

*Research Assistant to Dr. Lauren French* May 2013 - April 2014
*Allegheny College, Meadville, PA*

- Gained experience in general laboratory procedures, electrophysiology, non-mammalian vertebrate experimentation and molecular biology procedures
- Generated BK channel RNA using Northern blot, DNA and RNA isolation techniques
- Conducted recoverable surgeries with the non-mammalian model *Zenopus laevis* frogs and injected RNA using a nanoinjector into isolated *Zenopus laevis* oocytes
- Established electrophysiology procedures with *Zenopus laevis* oocytes using two electrode voltage clamp

**Grants**

*NASA West Virginia Space Grant Consortium* July 2018 – July 2019

- Awarded a 12,000 dollar grant to study the effect of a short and long-term dose of ethanol in Sprague Dawley rats on glucose absorption along the small intestine

**Scientific Conferences**

*41st Annual Scientific Meeting of the Research Society on Alcoholism* June 2018
*San Diego, CA*

- Presented a poster presentation on the novel effect of ethanol on glucose absorption along in intestinal epithelial cells among over 1,000 leading researchers in the field of alcoholism

*Digestive Diseases Week 2018* June 2018
*Washington D.C.*

- Presented a poster on the unique effect of ethanol on glutamine absorption in intestinal epithelial cells among over 14,000 leading researchers studying digestive diseases

*Digestive Diseases Week 2017* June 2017
*Chicago, IL*

- Attended the conference as a co-author on a poster describing the effect of the obesity on the Na-K-ATPase and another poster illustrating the effect of the adipose-derived secretome on glutamine absorption in intestinal epithelial cells

*Marshall University Health Sciences Research Day* August 2014 - 2019
*Marshall University Joan C. Edwards School of Medicine, Huntington, WV*

- Attended as a Marshall University Biomedical Research Graduate Student to share and discuss research conducted among colleagues at Marshall University

**Presentations**

*31st annual Marshall University Health Sciences Research Day* March 2019
*Marshall University Joan C. Edwards School of Medicine, Huntington, WV*

- Delivered a 15-minute presentation on the effect of moderate ethanol on glutamine absorption in an intestinal epithelial cell line (IEC-18)
- Awarded Best Oral Presentation

*27th annual Marshall University Health Sciences Research Day* March 2016
*Marshall University Joan C. Edwards School of Medicine, Huntington, WV*

- Delivered a 15-minute presentation on the use of intestinal stem cells, called enteroids, as a new model of intestinal research

**Committees**

*Ph.D. Student Admissions Interview Committee* January – April 2017, 2019
*Marshall University Biomedical Research Program, Huntington, WV*

- Actively participated in the interview committee for potential incoming Ph.D. graduate students by providing a current student perspective

*Administrative Director Interview Committee* July 2018
*Marshall University Biomedical Research Program, Huntington, WV*

- Provided a student's perspective during the interview process for the administrative director for the Marshall University Research Program

**Honors**

*Best Oral Presentation* March 2019
*Marshall University Joan C. Edwards School of Medicine, Huntington, WV*

- Placed first in the basic sciences category for students during Marshall University Health Sciences Research Day presenting on the effect of moderate ethanol on glutamine absorption in an intestinal epithelial cell line (IEC-18)

*Best Overall Performance as a Graduate Student* July 2017-2018
*Marshall University Biomedical Research Program, Huntington, WV*

- Awarded based on the overall research and academic performance as well as service through the Graduate Student Organization

*Poster of Distinction* June 2018
*Digestive Diseases Week, Washington D.C.*

- Received a Poster of Distinction award for a poster presentation on the effect of moderate ethanol on glutamine absorption in intestinal epithelial cells
- The award is distributed only among the top ten percent of accepted poster abstracts

*Graduate Student Organization Scholarship* August 2017
*Marshall University Biomedical Research Program, Huntington, WV*

- Received a 500 dollar scholarship from the Graduate Student Organization (GSO) based on scholastic merit, volunteer activities, and GSO participation

**Memberships**

*American Gastroenterological Association (AGA)* April 2018 – Present

- Hold a membership among 16,000 colleagues in the research, medical practice and advancement of gastroenterology

*Research Society on Alcoholism (RSA)* January 2018 – Present

- Maintain a membership among the leading scientists researching alcoholism and alcohol-related diseases

**Organizations and Activities**

*Biomedical Sciences GSO member* August 2014 - Present

- Hold a membership in the organization that represents the graduate students in the biomedical graduate program at Marshall University and conducts various community outreach, fundraising and volunteer activities

*GSO Secretary* May 2015 – May 2017

- Meeting and board meeting notes were taken and filed appropriately
- Attending all tutoring sessions to ensure questions by first year students were appropriately addressed

*GSO President* May 2016 – May 2017

- As President, all GSO board meetings and monthly meetings were planned accordingly. This includes managing the GSO's paperwork, funds and representing the GSO at Marshall University events

- Various fundraising activities were planned and organized including three bake sales, a soup sale, and a t-shirt drive
- Community outreach included planning and participating in the International Food Festival, Naloxone training, creating a GSO intramural sports team, local bowling nights as well as the Jared's box drive and the Loose the Training Wheels program
- Volunteer activities included tutoring sessions for Marshall University students and several presentations describing how to appropriately study for graduate school

*Marshall Women's Rugby Club* August 2014 – October 2018
*Huntington, WV*

- Member of the Marshall Women's Rugby Club
- Captain of the team from January 2018 through October 2018
- Initiated the first annual Prom on the Pitch fundraising match where all raised funds were donated to the Huntington City Mission Women and Family Shelter in April 2018

**Volunteer Activities**

*Jared's Box* December 2014, 2015, 2016 and 2017

- Gathered, wrapped and delivered toys for children staying at the local Hoop's Family Children's Hospital at Cabell Huntington Hospital and emergency room, St. Mary's Hospital and emergency room and the local Huntington City Mission Women and Family Shelter over the holidays
- Orchestrated the entire drive in December 2016 in which the organization had record donations from the graduate student organization in our area and expanded the donations to the Huntington City Mission Women and Family Shelter and St. Mary's Hospital Emergency Room
- Further expanded donations to the Cabell Huntington Hospital Emergency Room and St. Mary's Children's Ward in December 2017

*Tutor* September 2015 – May 2017

- Tutored biomedical research students in groups ranging from 3 to 20 in various biochemical topics and laboratory procedures before exams
- Organized tutors, locations and times for tutoring groups from September 2016 through the May 2017

*Science fair judge* December 2015

- Served as a science fair judge for the local Huntington high school
- Graded over 80 entries for their presentation skills, use of the scientific method, and overall project