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Effects of Inositol-6-Phosphate (Phytate) on Mucin 3 of the Gastrointestinal Tract

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Effects of Inositol-6-Phosphate (Phytate) on Mucin 3 of the Gastrointestinal Tract

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Abstract

Mucins are heavily glycosylated epithelial proteins. Under or overexpression of mucins may lead to several different types of dysfunctions. Mucins are under investigation as possible diagnostic markers for malignancies and other disease. This study examined the over/under expression of Mucin-3 in the presence of IP6. Three groups of six mice were fed varying levels of phytate (inositol 6-phosphate, IP6) for six days before being subjected to carbon dioxide asphyxiation. Sections of the duodenum, jejunum, ileum, stomach, and colon were collected, lysed, and the tissue proteins were prepared and analyzed by Western Blot to determine Mucin-3 expression. The hypothesis was that the presence of phytate in the digestive tract would modulate the expression of Mucin 3. Results showed that on average, Mucin 3 expression in untreated tissue was higher in areas of the duodenum and jejunum and lower in the ileum and stomach. Presence of IP6 decreased expression of Mucin 3 in the stomach by one percent in the 1g/kg phytate group while increasing in the 2g/kg phytate group by one percent. The expression of Mucin 3 in the duodenum increased fifty-five and thirty-nine percent in the 1g/kg and 2g/kg phytate groups, respectively. Jejunum Mucin 3 expression was increased by ninety percent in both 1g/kg and 2g/kg phytate groups. The ileum 0g/kg and 2g/kg phytate groups had the same expression of Mucin 3, but the 1g/kg group had a thirty-six percent decrease.

Introduction

Mechanism of Cancer and Anti-Cancerous Compounds

There has been a long search for compounds that can prevent cancers or inhibit the occurrence of certain cancers. Cancer generally arises through mutations in either stem or progenitor cells or mutations that alter a differential cell (Keyes, 2013). Most of the protective components of the cell include stimulatory (proto-oncogene) and inhibitory (tumor inhibition) components (Keyes, 2013). Cancer mechanisms include a signaling pathway that is specific for each gene. Alteration of this pathway allows cells to proliferate and multiply indefinitely (Keyes, 2013). A single oncogene is not sufficient to give rise to cancer, but over time, interactions between the environment and other proliferations give rise to more mutations (Keyes, 2013). Multiple molecular mechanisms, both intrinsic and extrinsic, converge to alter core cellular metabolism and provide support for the three basic needs of dividing cells: rapid ATP generation to maintain energy status; increased biosynthesis of macromolecules; and tightened maintenance of appropriate cellular redox status (Cairns, 2011). Mutations can alter the cell cycle checkpoints of the cell, which could lead to cancer. It is now clear that there are thousands of point mutations, translocations, amplifications and deletions that may contribute to cancer development, and that the mutational range can differ even among similar tumors (Cairns, 2011).

The cancer critical genes are often ultimately at the molecular level in the mitotic cell cycle at different checkpoints (G1, G2, and spindle assembly checkpoint) (Alberts, 2002). The G1 checkpoint checks for DNA damage, cell size, and growth factors. The G2 checkpoint also checks for cell size, and DNA replication. Alterations in these

checkpoints can lead to apoptosis, programmed cell death, which is critical for the cell to function properly (Alberts, 2002). Protein p53 is one of the proteins that control this critical function (Alberts, 2002).

Inositol Phosphates

Inositol 6-phosphate (IP6) or "phytate" is a compound found in starchy foods such as beans, cereal, nuts, legumes, pollen, and spores (Graf, 1990). The ability of phytic acid to complex with proteins and minerals has been a subject of investigation from chemical as well as nutritional standpoints (Urbano, 2000). Phytate is converted into compounds in the body that are used by cells to relay outside messages to the cell nucleus as well as aids in the catabolism of calcium and other minerals (Basic, 2010). Phytate as well as other inositol phosphates are abundant in mammalian cells (Singh, 2008).

Inositol phosophates function as second messengers for a variety of cell signaling mechanisms. Phosphorylation and dephosporylation of inositol phosphates are an important intracellular event of signal transduction in a variety of tissues (Yang, 1995). Second messengers are molecules that allow individual cells within the organism to respond to signals generated outside of the cell. An example would be plants in the field that respond to a variety of signals such as drought, pests and pathogens. Inositol phosphates have many defined roles in regulation in the cell. IP1 to IP5 are recognized as intracellular messengers. A second messenger role of inositol 1,4,5-trisphosphate is bringing about a host of cellular functions including mitosis *via* mobilizing intracellular Ca^{2+} is well recognized (Shamshuddin, 1995). Ca^{2+} sequestration is key function of 1,3,4,5-tetrakisphosphate (Shamshuddin, 1995).

Inositol 6-Phosphate

Both *in vivo* and *in vitro* experiments have demonstrated striking anticancer (preventive as well as therapeutic) effects of IP6 (Shamshuddin, 2002). Inositol 6-phosphate is a six-carbon sugar with six hydroxyl groups (inositol) that are phosphorylated (Shamsuddin, 2002). Inositol phosphates, including phytate play a critical role in neurotransmission (Shears, 1996), regulation of vesicle trafficking and recycling (Shears, 1996), and endocytosis and exocytosis (Effanov, 1997). Neurotransmisision is mediated by protein kinase C (Effanov, 1997), modulation of calcium influx coupled with the inhibition of phosphatases (Larsson, 1997), and efficient messenger RNA export (York, 1999 and Odom, 2000). IP_6 stimulates non-homologous DNA end joining of double strand DNA breaks by binding to the Ku70/80 subunits of DNA-dependent protein kinase *in vitro* (Hanakahi, 2002).

Inositol phosphates are synthesized from inositol and adults consume approximately 1g of inositol per day in their normal diets (Shamsuddin, 1996). Inositol phosphates are not just geared towards animals. Phytate, the salt of phytic acid, is widely distributed in the plant kingdom. It serves as a storage form of phosphorous and minerals and contains 75% of total phosphorous of corn kernels (Raboy, 2003). Other parts of plants such as roots, tubers and turions, are very low in phytate (Phillippy, 2003). Besides phytate, other inositol phosphates such as inositol pentaphosphates and inositol tertraphosphates are also present in seeds, however, to a much lower extent (Dorsch, 2003). During the germination of seeds, phytate is hydrolysed (Tabekhia, 1980 and Beal, 1985) and phosphate along with minerals such as calcium and magnesium becomes available for germination and development of the seedlings, explaining the significant

role of IP1-IP6. In addition to serving as the major phosphorus storage compound in seeds, inositol phosphates also act as natural antioxidants by chelation and reduction of the catalytic activities of many divalent transition metals (Verghese, 2006).

Phytic acid is one of the bioactive compounds that are being intensively studied to evaluate their effects on health. The chelation ability of phytic acid with minerals has been suggested to have beneficial effects toward lowering serum cholesterol and triglycerides and suppression of iron-mediated oxidation (Lee, 2005). It has been shown to have potential as an anticancer agent that only affects malignant cells and not normal cells and tissues (Shamsuddin, 2003). A variety of benefits of phytic acid on human health have also been reported including its potential as anti-cancer properties in soft tissue, colon, prostate, and mammary cancers. It may also act as an inhibitor for renal stone development (Dost, 2006 and Tokul, 2006). Phytate is not easily hydrolyzed. Ruminants readily digest phytate because of the phytase produced by rumen microorganisms (Klopfenstein, 2002). This compound is not obtained directly from the animal diet, but is synthesized inside the cell from phosphate and inositol, that is obtained in the diet (Hanakahi, 2000). The interaction of intracellular phytic acid with specific intracellular proteins has been investigated *in vitro*, and these interactions have been found to result in the inhibition or potentiation of the physiological activities of those proteins which could lead to certain anti-cancerous benefits (Hanakahi, 2000 and Norris, 1995).

Mucin 3

Mucin 3 (Muc-3) is a large glycoprotein expressed by many tissues in the body including the stomach, intestine, gallbladder, and colon (Weiss, 1996). A sequence of tandem repeats in the structure can be identified through cloning and characterization of intestinal cDNA using a probe. There are seventeen residue threonine and serine tandem repeats in the domain. The gene for Muc-3 is located at position q22 (long arm) on chromosome seven. Muc-3 expression differs from other mucin proteins in the human small intestine as it is expressed in both goblet cells and enterocytes while others are specifically expressed in goblet cells (Gum, 1997). Muc-3 is a trans-membrane glycoprotein that is seen in both colorectal cancers and normal colon (Chang, 1994) with a 3′ terminus coding for two epithelial growth factor-like domains, a transmembrane domain and a cytoplasmic tail (Mack, 2003). Studies have shown an association between Muc-3 expression and poor prognosis in a number of cancers including pancreatic (Park, 2003), breast (Rakha, 2005), gastric (Wang, 2003) and renal (Leroy, 2003). There is some evidence suggesting that Muc-3 expression is reduced in colorectal cancers and that this varies between histological types (Weiss, 1996).

Mucins and cancer

Mucins have been implicated in the pathogenesis of certain cancers such as adenocarcinomas and malignant tumors of the intestinal tract. It has been proposed that mucins act as markers for tumors to map the microenvironment during invasion and growth. Immunohistochemical studies have tumor-associated antigen sites on mucins. These sites induce a conformational change when over/under glycosylated. A decrease in

glycoprotein mucin in serum has shown proportional correlation to cancer growth and tumor burden. This leads to the suspicion that abnormal forms of mucin might lead to certain cancers (Hollingsworth, 2004). The relationship between IP6 and mucin expression has not been exhaustively shown. Experiments that demonstrated the anticancer feature of phytate were performed by Shamsuddin et al. (1998) and concluded that only diets containing high phytate content (cereals and legumes) showed a negative correlation with colon cancer.

Dietary phytates occur in the digestive tract and they come in contact with mucins that line the digestive tract such as the transmembrane Muc-3. The consequence and role of such interactions is yet to be discovered. We hypothesized, in the present study, that the presence of phytate in the digestive tract will alter the expression of Muc-3. To test the hypothesis, the expression of Muc-3 in gastrointestinal tissues in the presence of phytates will be determined.

Materials and Methods

Eighteen 8-week old black-6 (C57BL/6) mice were appropriately acclimatized for 3 days before experimentation. They were housed in cages in groups as littermates. Mice were then allowed to settle down in their new cages for 3 days before experimentation. Mice were fed a commercial regular diet. Different cages were randomly divided into 3 groups that were assigned to the 3 different treatments (IP6 dose rates of 0, 1, and 2 g/kg body weight). Each treatment group consisted of 6 mice. IP6 for dosing was prepared in 10% sucrose solution by preparing 100 mL of 80 g of IP6/100 ml solution (Na phytate salt was used, Sigma-Aldrich Corp., St Louis, MO, USA, Catalog # P8810) and then serial diluted to obtain 40 g and 20 g IP6/100 mL solutions. In order to dose a fixed volume of treatment material per gram of body weight different concentrations of IP6 were used: 40 g IP6/100 mL solution was used for the 2 g IP6/kg BW dose rate; 20 g IP6/100 mL solution was used for the 1 g IP6/kg BW dose rate. The same volume of 10% sucrose solution was used for the control animals (0 g IP6/kg BW dose rate). Hence, for a given bodyweight, the same volume of treatment material was dosed (5 mL/kg BW).

The 3 treatment groups of mice were dosed orally with IP6 solution using gavage needles for 5 days. Mice were weighed once at the beginning of the experiment before the dosing and also on day 6 before they were euthanized by carbon dioxide asphyxiation. The gastrointestinal tract was dissected and divided into different sections: stomach, duodenum, jejunum, ileum and colon. The samples were placed in dry ice after collection and subsequently stored at -80 °C for later use or kept on ice for immediate homogenization.

Preparation of Lysate From Tissues After Storage

To prepare the lysate, lysis buffer was added to each tube of tissue and the tissue homogenized with an electric homogenizer for two minutes, and centrifuged twice for 20 minutes at 16000 x g and 4°C in a micro centrifuge. The tubes were placed in ice and the supernatant was transferred to another tube while discarding the pellet. Samples were diluted to 2 μg/mL for SDS-PAGE.

BSA Protein Assay Technique

A BSA protein assay was used to determine the correct concentration of protein to dilute for SDS-PAGE standards (2ug/mL) in conjunction with a 96 well plate. Pierce™ Bovine Serum Albumin Standards were used according to manufacturer instruction to make protein standards. The albumin standard was formulated at 2mg/mL in ultrapure 0.9% sodium chloride (saline). Standards are used to generate a standard curve for known protein concentrations at different concentrations to measure against the sample. The next step is to dilute proteins to a 1:1000 ratio. 25 uL of each standard (A-I) were added to each well in the first row of the 96 well plate. The samples were diluted 1:1000. The plate was then incubated in a water bath for 30 minutes. A NanoDrop 2000/2000c Spectrophotometer was used to determine protein concentration.1 uL sample of protein from each mouse was placed on the NanoDrop 2000/2000c-testing platform after setting a standard curve using BSA standards.

Gel Electrophoresis

SDS-PAGE is a form of gel electrophoresis that can separate proteins based on molecular weight exclusively. SDS (Sodium Dodecyl Sulfate) is a reagent that denatures proteins (disrupts secondary, tertiary, and quaternary structure) and gives them a uniform negative charge. The proteins can migrate towards the anode of the apparatus with smaller proteins traveling farther. The gel was placed in casting stands making sure the wells face the inside of the chamber. The tank was then filled 1x chamber buffer to maintain pH upon separation with electrode wires attached to their correct charge. The volume of protein (2 ug/uL) to be loaded in the well was calculated using the dilution formula C1V1=C2V2 and gel was loaded after heating the proteins at 95 °C for four minutes. 10 uL of marker and 20 uL samples were used. The gel was run at 200V and 40 mA for 60 minutes.

Transfer of Proteins to Membrane

To transfer the proteins to membrane, the gels were removed and placed in transfer buffer while assembling the gel transfer apparatus in the following order: sponge, filter paper, gel, membrane, filter paper, and sponge. The order is important to ensure the protein gel is correctly applied to the membrane after transfer. The PVDF membrane was soaked in methanol before assembling. The highly hydrophobic nature of the PVDF membrane would inhibit transfer of the gel if methanol were forgotten. The chamber was filled with transfer buffer up to blocking line and an ice block was added as well as a stir rod before running at (200V and 350mA for 60 minutes).

Blotting

The membrane was removed and gently put in a box with 5% skim milk overnight at 4 °C. The skim milk was made by dissolving 4.54 g of casein with 100 mL of 1x tris-buffered saline with tween (TSBT) to maintain pH while blotting. The milk will bind to proteins that are not of interest. The membrane was then taken and washed 3x with 1x TBST for 15 minutes each. Tween, a component of TSBT, attaches to the membrane elsewhere than the target protein. When antibody is added, there is no place for the antibody to bind other than target protein, which results in fewer false positives and clearer results. Two vials of the primary antibody were added (8 uL) to 8mL of 5% skim milk. The primary antibody will bind to the epitope region of the protein. The membrane was stored overnight at 4°C. The membrane was then washed 3x with 1x TBST again for 15 minutes to rid the membrane of milk before adding the secondary antibody. Secondary antibody was added at 6uL to 9mL milk (1ul to 1.5 mL ratio), and the membrane was placed in the secondary antibody solution at room temperature for one hour. The luminescent secondary antibody will bind to the protein-primary antibody complex. Once the substrate is added, a reaction occurs that fluoresces the membrane. The membrane was washed again 3x with 1x TBST for 15 minutes each before observed under chemiluminescence using Thermo Scientific™ SuperSignal™ West Femto Chemiluminescent Substrate Kit.

Chemiluminescence

The membrane was treated with SuperSignal™ mix (substrate of reaction). This was accomplished by mixing 1mL of Reagent A with 1mL of Reagent B to give 1:1 ratio to give enough to use on one membrane. The light that is detected is a transient product of the reaction when the protein-antibody-enzyme substrate complex binds and the reaction occurs. The membrane was visualized with a Proteinsimple™ FluorChem E Imager under UV light.

Results and Discussion

The final body weight of the mice fed with 2g/kg phytate was higher than mice fed with 1g/kg and 0g/kg, respectively, as shown in Table 1. The 2g/kg phytate group was the sole group that gained weight during the study (figures 1 and 2). This could be possibly due to phytate influencing the uptake of nutrients. According to Panda (2005), chickens that were fed a diet of non-phytate phosphorus had decreased weight compared to the group that had been given phytate phosphorus.

Group	Average starting weight (g)	Average final weight (g)	Average weight change (g)
0 g/kg group	**23.1**	**20.0**	**-3.1**
1 g/kg group	**22.0**	**20.2**	**-1.8**
2 g/kg group	**23.4**	**25.0**	**+1.6**

Table 1: Average body weight of mice fed different levels of phytate

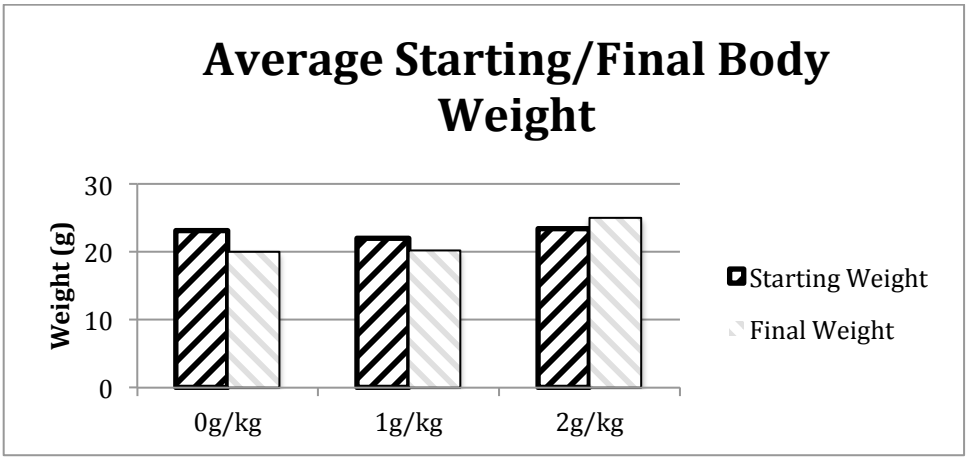

Figure 1: Average starting and final body weight of mice fed with different levels of phytate

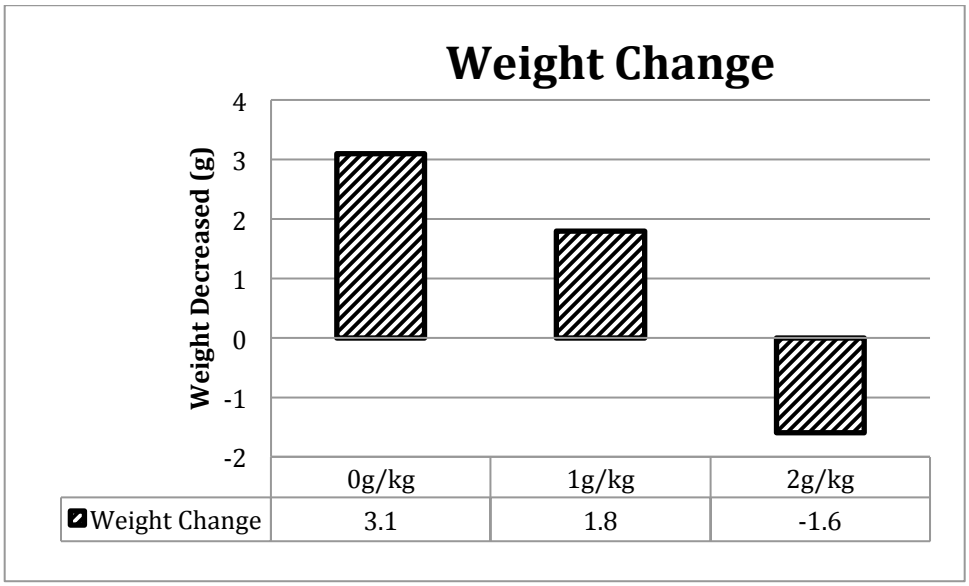

Figure 2: Average change in body weight of mice fed differing levels of phytate

Expression of Muc-3 changed along the gastrointestinal tract (figure 3), which showed decreased levels along the length of the small intestine. The lowest expression of Muc-3 was found in the stomach.

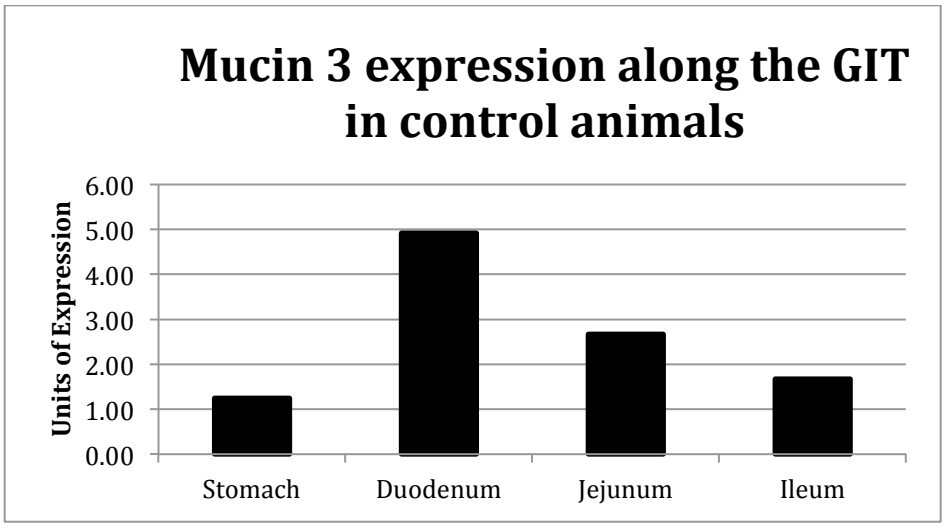

Figure 3: Expression of Mucin 3 along the gastrointestinal tract in control

Mucin 3 expression in the stomach and the intestinal sections is shown in figures 4-7. Different doses of IP6 resulted in an increased expression of Muc-3 primarily in the duodenum (Table 2, Figure 4) and jejunum (Table 3, Figure 5), however, the expression decreased in 1g/kg stomach (Table 4, Figure 6) and ileum (Table 5, Figure 7) tissue.

Duodenum	Well 2 0g/kg 1,2	Well 3 0g/kg 3,4	Well 4 0g/kg 5,6	Well 5 1g/kg 1,2	Well 6 1g/kg 3,4	Well 7 1g/kg 5,6	Well 8 2g/kg 1,2	Well 9 2g/kg 3,4	Well 10 2g/kg 5,6
Area of Expression	48	936	N/A	592	777	297	252	1092	660

Table 2: Mucin 3 expression in mouse duodenum tissue

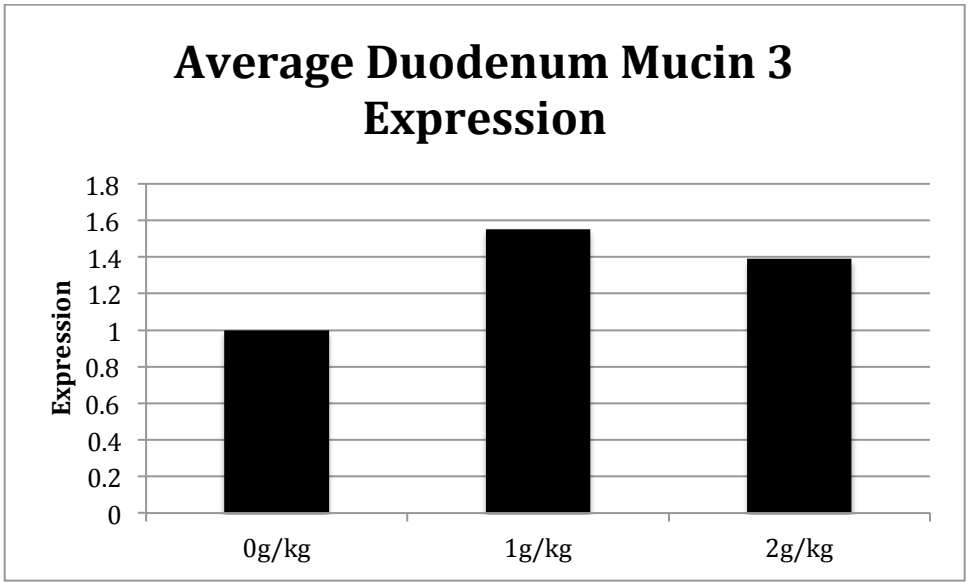

Figure 4: Mucin 3 expression in duodenum

Jejunum	Well 2 0g/kg 1,2	Well 3 0g/kg 3,4	Well 4 0g/kg 5,6	Well 5 1g/kg 3,4	Well 6 1g/kg 5,6	Well 7 2g/kg 1,2	Well 8 2g/kg 3,4	Well 9 2g/kg 5,6
Area of expression	120	N/A	132	348	N/A	444	420	168

Table 3: Mucin 3 expression in mouse jejunum tissue

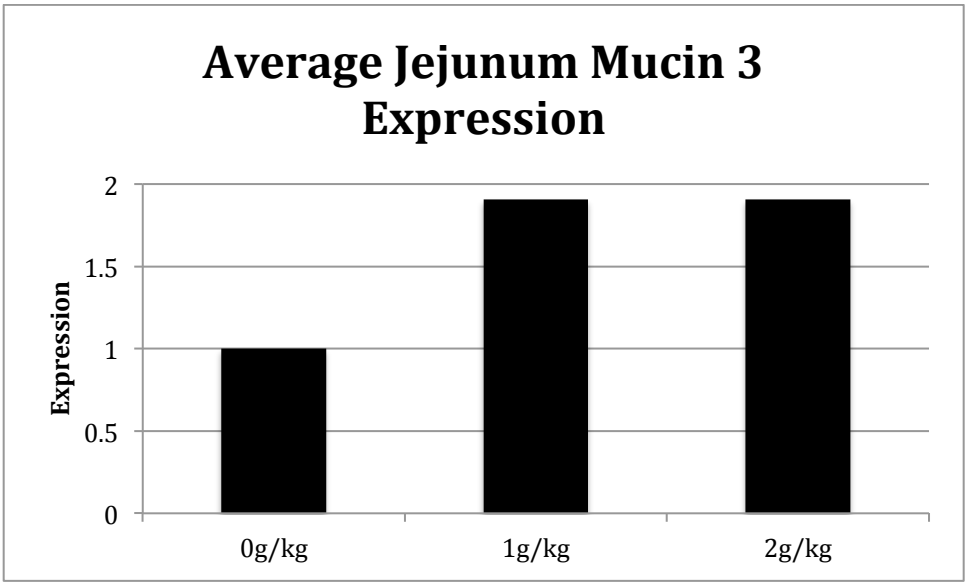

Figure 5: Mucin 3 expression in jejunum

Stomach	Well 2 0g/kg 1,2	Well 3 0g/kg 3,4	Well 4 0g/kg 5,6	Well 5 1g/kg 1,2	Well 6 1g/kg 3,4	Well 7 1g/kg 5,6
Area of Expression	136	122	116	132	120	52

Table 4: Mucin 3 expression in mouse stomach tissue

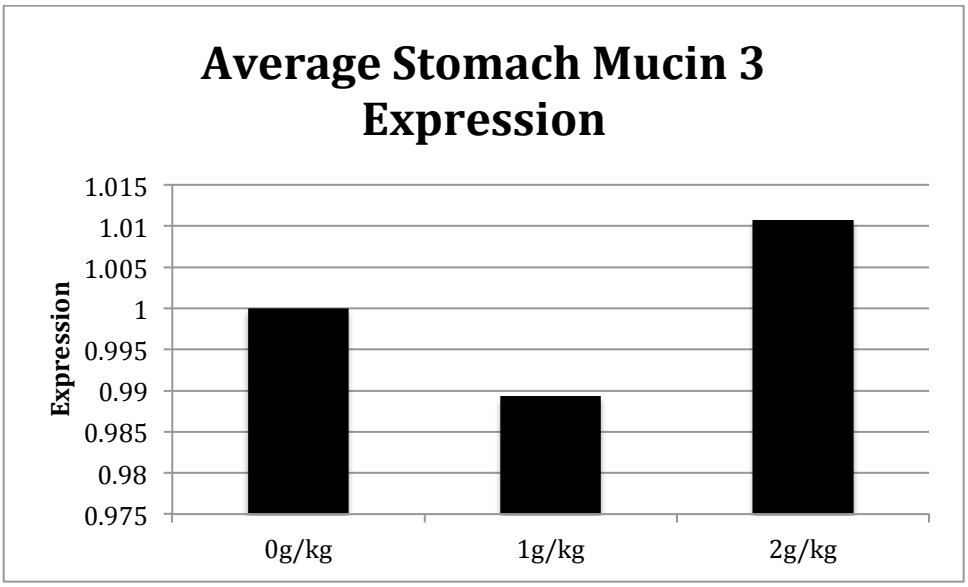

Figure 6: Mucin 3 expression in stomach

Ileum	Well 2 2g/kg 1,2	Well 3 2g/kg 3,4	Well 4 2g/kg 5,6	Well 5 1g/kg 1,2	Well 6 1g/kg 3,4	Well 7 1g/kg 5,6	Well 8 0g/kg 1,2	Well 9 0g/kg 3,4	Well 10 0g/kg 5,6
Area of Expression	420	108	272	132	396	310	546	N/A	189

Table 5: Mucin 3 expression in mouse ileum tissue

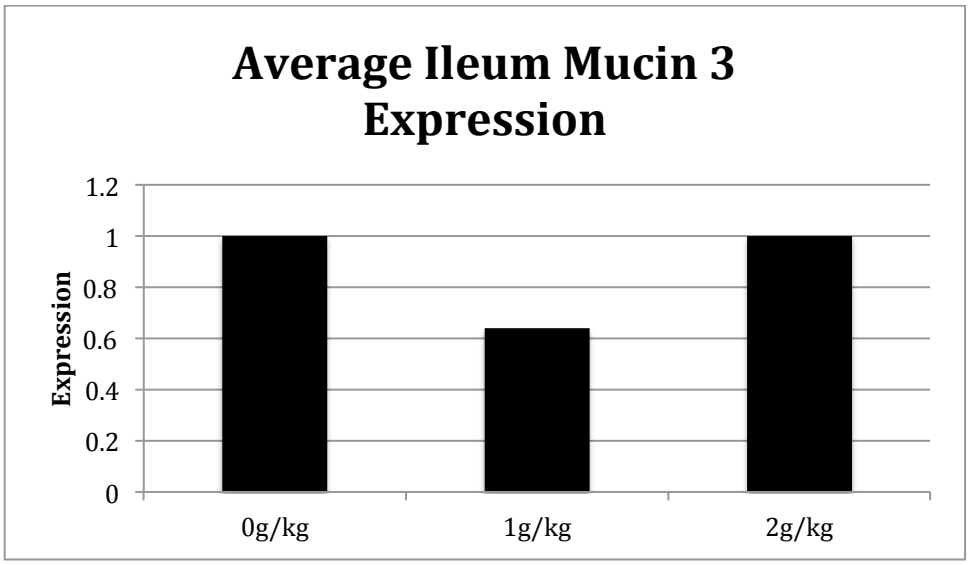

Figure 7: Ileum Mucin 3 expression

The increased expression of Muc-3 in the duodenum (Figure 4) and jejunum (Figure 5) could be due to inositol phosphate increasing goblet cell production. Satchithanandam (1990) observed that when mice are fed diets supplemented with inositol phosphate, they had higher goblet cell activity, higher cytokine production, and greater incorporation of radioactive tracers. Goblet cell production also can increase Muc-3 production.

There was increased expression from the 0g/kg phytate group to the 2g/kg phytate group in all protein Muc-3 tissue. The Muc-3 expression from the 2g/kg phytate group in the stomach and ileum remained the same or decreased from the control (Table 2 and Table 5). This could be due to IP6 interacting strongly with Muc-3 in those areas (Kufe, 2015). A loss of mucin may confer a microenvironment in which bacteria can activate an inflammatory response at the epithelial surface (Kufe, 2015). A decrease in glycoprotein mucin in serum has shown proportional correlation to cancer growth and tumor burden

(Hollingsworth, 2004). A decrease in glycoprotein mucin could also lead to increased inflammatory/growth responses in the stomach and ileum region due to less/normal mucin expression in the 1g/kg and 2g/kg groups due to phytate.

As shown in Table 6, the area of expression of Muc-3 stayed relatively the same in the stomach tissue.

Average Expression	Duodenum	Jejunum	Ileum	Stomach
0g/kg	492	126	266	124
1g/kg	764	240	170	123
2g/kg	685	240	266	126

Table 6: Average expression of muc-3 in duodenum, jejunum, ileum, and stomach tissue

Mucin content in the stomach is highly protected due to acidic pH in stomach. In fact, according to Boltin (2013), fifty percent of gastric lesions in gastric ulcer patients had the same mucin content as stomach tissue without lesions. Excessive ingestion of undegraded phytates can cause mineral deficiencies in humans (Iqbal, 1994). The ileum absorbs fat-soluble vitamins and minerals. A decrease of expression in the 1g/kg and 2g/kg phytate groups compared to the control as shown in Table 5 may correlate to Muc-3 decrease of expression due to phytate disturbing the uptake of minerals and vitamins.

In summary, IP6-mediated expression of Muc-3 in the stomach stayed relatively similar. The expression of Muc-3 in the duodenum increased by an average of forty-seven percent in the 1g/kg and 2g/kg phytate groups when compared to the control group. Jejunum Muc-3 expression was increased by ninety percent in both 1g/kg and 2g/kg

phytate groups. The ileum 0g/kg and 2g/kg phytate groups had the same expression of Muc-3, while the 1g/kg phytate group showed a thirty-six percent decrease. In conclusion, Muc-3 expression in the presence of IP6 did not change in the stomach, increased in the duodenum and jejunum, and decreased in the 1g/kg dose in the ileum. Hence our hypothesis that the presence of phytate in the digestive tract will alter the expression of Muc-3 was proven to be true in the duodenal and jejunal sections of the gastrointestinal tract. The effects of IP6 may be through changing the expression of Muc-3 in the duodenum and jejunum.

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