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Protection against Gluten-mediated Tight Junction Injury with a Novel Lignite Extract Supplement

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Abstract

Background: Tight junctions are found in epithelial cells and function as selective gatekeepers to regulate absorption. PT-gliadin is the gluten protein segment that is known to impair the functioning of tight junctions. This study aimed to examine the effects of a lignite extract dietary supplement (RESTORE) on tight junction function in small intestine (IEC-6) and colon (Caco-2) epithelial cells. The study also evaluated the biologic safety of the same supplement as established by the rates of apoptosis in the intestinal and proximal renal tubule cell cultures treated with the supplement.

Methods: IEC-6 and Caco-2 cells were incubated until a stable trans-epithelial electrical resistance (TEER) was measured. The dietary supplement at 20% concentration or a control were placed on the cells and left overnight. These cells were then treated with and without PT-gliadin. Tight junction expression was determined by immunofluorescent microscopy. The rate of apoptosis was established in cell culture with the lignite extract at 20% concentration in order to assess a toxic concentration in normal cell lines: IEC-6, Caco-2, and human renal proximal tubule cell (RPTC) lines.

Results: The lignite extract supplement increased the TEER in IEC-6 (58%) and Caco-2 (15%) compared to control. PT-gliadin dramatically decreased the TEER in both control IEC-6 (49%) and control Caco-2 (27%) membranes. The lignite supplement prevented PT-gliadin-mediated decrease in TEER. The supplement reduced apoptosis in RPTC (44%), IEC-6 (13%), and Caco-2 (24%) cell cultures.

Conclusion: The lignite supplement blocked a PT-gliadin dependent decrease in TEER in small intestine and colon cell line membranes. The lignite extract was not toxic on intestinal or renal cells at high concentration, and demonstrated a statistically significant reduction in apoptosis in RPTCs. Human clinical trials are needed to evaluate the use of RESTORE to support health in gluten-sensitive individuals.

Keywords: Lignite; Transepithelial electrical resistance (TEER); Caco-2; IEC-6; Gluten sensitivity; Zonulin; RESTORE; PT-gliadin; Tight junctions; Renal proximal tubule cell (RPTC)

Introduction

Tight junctions are expressed by epithelial and endothelial cells to form the macro membranes of the digestive tract, vascular system, and the blood-brain barrier. These tight junctions function as selective gatekeepers that regulate the absorption of macronutrients, and compose a frontline of defense. The increased gut permeability that results from tight junction dysfunction is increasingly recognized as an early step in the pathogenesis of many acute and chronic inflammatory diseases, including celiac disease and inflammatory bowel disease (Crohn's Disease and ulcerative colitis) [1-6]. The chronic inflammatory underpinnings of these conditions point to the chronic immune system activation of the gastrointestinal-associated lymphoid tissue that becomes exposed with tight junction dysfunction.

Gliadin is a component of gluten created during digestion that is known to impair the functioning of tight junctions via the zonulin occludin pathway. The common syndrome of gluten sensitivity now affects more than 18 million individuals in the US alone. Celiac disease, an autoimmune reactivity to gliadin, also affects a rapidly growing number of individuals worldwide. The rapid rise of these epidemics over the last 30 years raises the possibility of a progressive, widespread biologic shift in the human intestinal microenvironment.

Healthy soil, similar to a healthy human intestinal ecosystem, contains a vast library of nutrients, minerals, amino acids, and other complex metabolites that are released through the digestive processes of bacteria and fungi. As the nutrient density has waned in the soils of our modern agricultural system, health practitioners around the world have increasingly turned to fossil soil (lignite) extracts to supplement human nutrition. Naturally-oxidized lignite extracts including shilajit, humic acids, and fulvic acids have been used as dietary supplements to deliver soil-based minerals and amino acids in China and India for hundreds of years. Their clinical use has been limited by their oxidative nature, frequent contamination by pathogenic bacteria, or inorganic

chemicals in these acidic compounds. Significant contaminants that occur during mining and manufacturing of these products in India and China have been discovered to be present in numerous commercial sources [7,8].

RESTORE is the first lignite-derived dietary supplement that delivers a stabilized family of carbon-based redox molecules as the active ingredient resulting in an alkaline liquid form that carries only trace minerals and amino acids. While various clinical trials have been performed with some lignite-containing compounds, rigorous testing of standardized extracts is needed to better establish the different biologic effects and safety of these distinct classes of lignite extracts [9].

In this study, we examine the biologic effects of RESTORE lignite extract on the tight junction barrier system of the gut via trans-epithelial electrical resistance (TEER) of polarized epithelial membranes of normal small bowel epithelium cells (IEC-6) and a colon adenocarcinoma cell line that retains many characteristics of normal colon epithelium cells (Caco-2) [10-12]. To our knowledge, there are no previously published studies looking at lignite extract effects on polarized epithelial cell lines as performed in this study. Additionally, no lignite extract has been shown to be protective against a known and prevalent intestinal toxin, PT-gliadin, an element of gluten. Because gluten has been implicated as a causative agent in the pathophysiology of celiac disease as well as in non-celiac gluten sensitivity, we examined whether the lignite extract had protective effects toward this pervasive and common food-borne toxic compound on intestinal epithelial cell model systems [13,14].

The use of primary cultured renal proximal tubule cells (RPTCs) for toxicity to xenobiotics is a well-established method for *in vitro* testing, and therefore was used in the safety testing of the lignite extract supplement [15]. Cells in culture show a constant, but low level of apoptosis, and if a xenobiotic has even low levels of toxicity, the rate of apoptosis is increased. Therefore, a sensitive-flow cytometric assay for apoptosis was chosen for testing RESTORE human RPTC lines.

Methods

Cell culture

Human colorectal carcinoma (Caco-2) and rat ileum epithelial (IEC-6) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA; ATCC catalog HTB-37 [Caco-2 cells]); ATCC catalogue CRL-1592 [IEC-6 cells]). Both cell lines were propagated in their respective specific media according to manufacturer protocols.

Primary cultured human renal proximal tubule cells (RPTC, Lifeline), were the third polarized epithelial cell type used and were propagated in its media according to manufacturer protocols. During experiments each of the cells were cultured in the same media, Dulbecco's Modified Eagle Medium (DMEM-F12, Invitrogen, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) at 37°C in 5% CO_2 with and without treatments.

Cell viability

Apoptotic cells were measured by incubation with Alexa 647 Annexin V (Invitrogen Waltham, MA, USA) in suspension and measured by flow cytometry. Cells were trypsinized and incubated with Annexin V according to the manufacturer's protocol and measured using a BD Accuri Flow Cytometer in 96 well plates using the autosampler. Unstained and unlabeled cells were used for background gaiting and apoptotic cell positive control cells were identified by incubation with 10 μM staurosporine (Sigma-Aldrich, St. Louis, MO, USA). Three different polarized epithelial cell types were exposed to the lignite extract supplement at a 20% vol/vol concentration in media for 18 hours and tested for Annexin V binding as a measure of toxicity via induction of apoptosis. A 20% concentration was used because it was the concentration which is considered a maximal dose to determine if apoptosis is increased due to toxicity.

RESTORE itself is comprised of the 150 mg of Terrahydrite™ lignite extract at pH 8.7 and is therefore not considered fulvic or humic acid. The supplement also has purified water, as well as a proprietary blend the following ingredients, which comprise less than 1500 ppm: (inorganic compound) chloride, sodium, lithium, calcium, phosphorus, sulfur, bromide, potassium, iron, antimony, zinc, copper, gold, magnesium, (organic compounds) alanine, glycine, histidine, isoleucine, methionine, threonine, and valine.

Exposure of cells to gluten

One gram Gliadin (G3375, Sigma-Aldrich, St. Louis, MO, USA) was digested with 20 mg Pepsin (7012, Sigma-Aldrich, St. Louis, MO, USA) in 10 ml 100 mM HCl for two hours, then adjusted to pH 8.0 with 5 M NaOH, then 20 mg Trypsin (8642, Sigma-Aldrich, St. Louis, MO, USA) digested over four hours at room temperature. The trypsin was heat inactivated at 90°C for three minutes. The PT-Gliadin peptides responsible for tight junction disassembly-QVLQQSTYQLLQELCCQHLW (151-170) and QQQQQQQQQQQQILQQILQQ (111-130)-that bind to CXCR3 and release zonulin have been previously identified [2].

Trans-epithelial electrical resistance

Trans-epithelial electrical resistance (TEER) was measured in Caco-2 and IEC-6 cells seeded in 24-well transwell plates (1 micron pore size, Becton Dickinson), incubated until a stable TEER was measured three days in a row. TEER was measured using the epithelial Volt-ohmmeter fitted with a planar electrode (World Precision Instruments). Water alone or water with RESTORE were used to make media from powder (DMEM-F12, Invitrogen), and were placed on the cells and incubated at 5% CO_2 in a humidified 37°C incubator for the appropriate amount of time. Peptic tryptic digest of gliadin (G3375, Sigma), was made according to standard protocol, and added to apical media at 1 mg/ml. Measurements were made at the two-hour time point [16,17].

Zona occludens protein 1 immunofluorescence microscopy

Directly following TEER measurements, IEC-6 cells were simultaneously fixed and permeablized in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) 1% Triton X100 (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 5 minutes. Cells were washed and then blocked in 2% bovine serum albumin (BSA) and incubated with 1 to 20 dilution of cell culture supernatant from Hybridoma clone R26.4c, producing anti-ZO1 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Alexa 488 labeled donkey anti-mouse IgG (Invitrogen, Waltham, MA, USA) was used at 4 μg/ml to make the antibody fluorescent. Nuclei were stained with Hoechst 5 μg/ml. Cells were imaged with a Zeiss (Oberkochen,

Germany) Axiovert automated 6 D fluorescent microscope and 100 × 1.4 NA plan apochromatic objective.

Statistics

All experiments were run five times and values shown are results of each of the five. Data are presented as mean values ± the standard error from the mean. P-values were obtained by performing one-way analysis of variance between groups.

Results

The two bowel-derived polarized epithelial cell lines, IEC-6 and Caco-2, were able to consistently form a stable, electrically resistant barrier on transwell inserts, and thus were tested for changes in TEER when exposed to the lignite extract supplement. In both of these cell types the TEER was significantly increased in the presence of RESTORE in comparison to the VEH (Figures 1 and 2) or vehicle which is the carrier compound used to make the stock solution and is used as a control in the experiment. In IEC-6 cells the TEER was increased by 58%, n=8, p<0.05. In Caco-2 cells the TEER was increased by 15%, n=4, p<0.01.

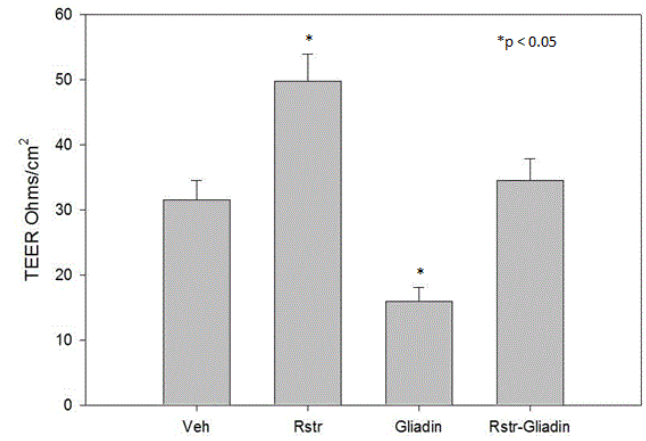

Figure 1: Effects of PT-gliadin (1 mg/ml) and RESTORE (Rstr) lignite extract (20% vol/vol concentration in media) on the trans-epithelial electrical resistance (TEER) of IEC-6 monolayers. Data are presented for two independent experiments, with four replicates each. Results are written as mean ± standard deviation [*represents a TEER value that is statistically significantly different from the TEER of the vehicle (p<0.05)].

When testing the lignite extract's effect in conjunction with a known intestinal barrier toxin, PT-gliadin, PT-gliadin was found to decrease TEER in IEC-6 by 49%, n=4, p<0.05. The lignite supplement completely blocked PT-gliadin's decrease on TEER, n=4, p<0.05. Likewise, PT-gliadin decreased TEER in Caco-2 by 27%, n=4, p<0.05. Again, the lignite extract completely blocked PT-gliadin's decrease on TEER, n=4, p<0.05.

A known mechanism by which PT-gliadin has been shown to decrease TEER in IEC-6 and Caco-2 cells is by disruption of tight junctions [18]. The effect of the lignite extract and PT-gliadin on tight junction formation is seen by examining the tight junction localization of ZO1 by immunofluorescence microscopy in IEC-6 (Figure 3).

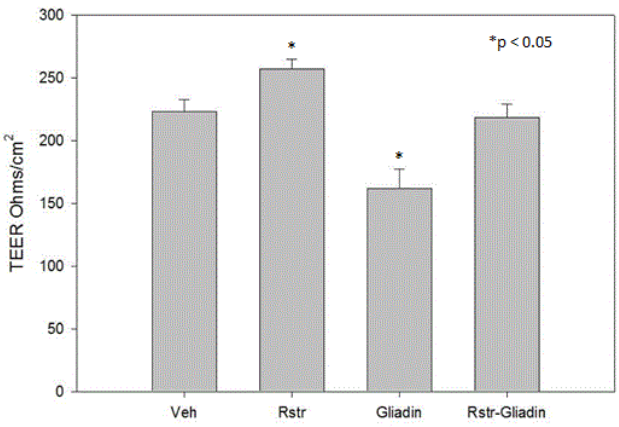

Figure 2: Effects of PT-gliadin (1 mg/ml) and RESTORE (Rstr) lignite extract (20% vol/vol concentration in media) on the trans-epithelial electrical resistance (TEER) of Caco-2 monolayers. Data are presented for two independent experiments, with four replicates each. Results are written as mean ± standard deviation [*represents a TEER value that is statistically significantly different from the TEER of the vehicle (p<0.05)].

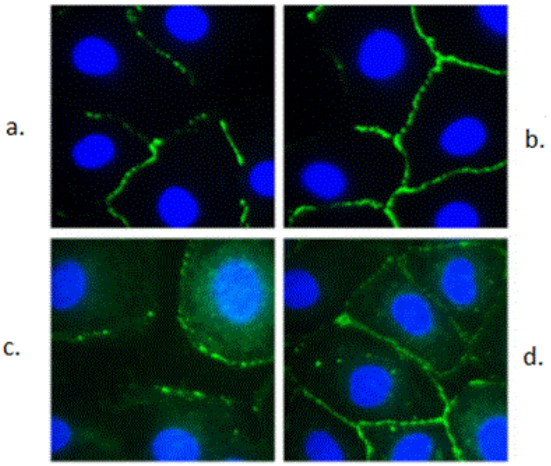

Figure 3: ZO1 immunofluorescence of IEC-6 cells. The cells were grown and then exposed to Hybridoma clone R26.4c to produce anti-ZO1 monoclonal antibody. Alexa 488 labeled donkey anti-mouse IgG was used at 4 µg/ml to make the antibody fluorescent. Nuclei were stained with Hoechst 5 µg/ml. Cells were imaged with a Zeiss (Oberkochen, Germany) Axiovert automated 6 D fluorescent microscope and 100 × 1.4 NA plan apochromatic objective. Images were taken of (a) the vehicle, (b) exposure to lignite extract (20% vol/vol concentration in media) only, (c) exposure to PT-gliadin (1 mg/ml) only, and (d) exposure to both lignite extract (20% vol/vol concentration in media) and PT-gliadin (1 mg/ml).

In VEH control cells the localization of ZO1 shows intermittent gaps in ZO1 between cells (a). When RESTORE was incubated with cells for only two hours, an increase in abundance of ZO1 between

cells can be visualized as represented by (b). PT-gliadin dramatically decreased the continuity of ZO1 localization between cells (c), and RESTORE prevented this loss of ZO1 localization between cells (d).

In all three cell types exposed to the lignite extract supplement and tested for toxicity, there was no increase in apoptosis (Figure 4) when measured for Annexin V binding by flow cytometry. In both IEC-6 (13%) and Caco-2 (24%) cells there was a trend toward lower apoptotic rates, but these did not reach statistical significance. In RPTCs there was a statistically significant decrease in the rate of apoptosis (-44 ± 5%; N=4; $p<0.001$).

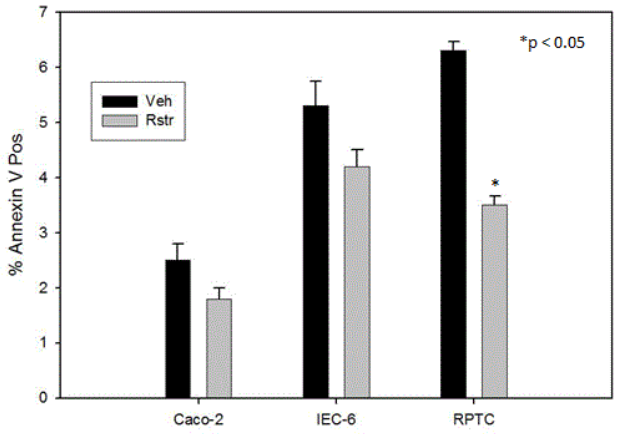

Figure 4: Effects of lignite extract (20% vol/vol concentration in media) on apoptosis of IEC-6, Caco-2 and RPT cells was measured incubation with Alexa 647 Annexin V in suspension and measured by flow cytometry. Annexin V binding as a measure of toxicity via induction of apoptosis with (grey bars) and without (black bars) lignite extract (Rstr) [*represents a value that is statistically significantly different from the vehicle ($p<0.05$)].

Discussion

Intercellular tight junctions are an integral part of forming a functional polarized epithelial layer and allowing vectorial transport of water and electrolytes across the intestinal epithelium. The anatomical and functional arrangement of the gastrointestinal tract regulates passage of micro-and macro-molecules between the environment and the host through transcellular transport (micromolecules) and paracellular diffusion (macromolecules) via modulation of the intercellular tight junctions. To prevent harm to the host and reduce inflammation, a fully functional paracellular pathway minimizes antigen presentation and toxin exposure of the gut-associated lymphoid tissue (GALT) adjacent to the bowel epithelium. These tight junctions, also called zonula occludens, form a regulatory barrier throughout the digestive tract that acts as an active transport pathway of macronutrients into the body, and a firewall against unwanted toxins and host pathogens [19].

A growing number of manufactured and naturally-occurring elements in processed foods and mono-crop farming are being implicated in tight junction damage [20]. In the developed world, the unintentional chronic stimulation of zonulin-mediated intestinal permeability from food elements, such as the gluten-derived peptides that include the PT-gliadin used in this study, compromises tight junction integrity and leads to unregulated absorption of organic and inorganic material. Gluten is a protein found in foods processed from wheat and other related grains (e.g. kamut, barley, and rye). The quantity of refined gluten products has markedly increased in the American diet in recent decades, and the rates of clinically-recognized gluten sensitivity and allergy are rapidly on the rise. The clinical manifestations of gluten sensitivity illustrate the chronic inflammatory repercussions of gluten-mediated membrane permeability, with symptoms including arthralgia, fatigue, cognitive deficits, irritable bowel, neurologic dysfunction, and chronic pain [21,22]. The findings in this study demonstrate a common mechanism by which gluten-mediated tight junction damage can predispose the host to unregulated antigen presentation at the GALT.

There are only a handful of studies that have shown improvements in tight junction formation and trans-epithelial electrical resistance of polarized epithelial cells including small and large intestine cells. Some of these substances include the bioflavonoid quercetin and indole, butyrate, nicotine, the amino acid L-glutamine, the mineral zinc, the pharmaceutical compound and zonulin-inhibitor larazotide, and now the novel lignite extract studied here [19-30]. The lignite extract supplement has unique biologic effects among these reported compounds in both the speed of response in the TEER functional analysis of the tight junction, which occurred within 60 min from introduction to the membranes, and the extent of the response in regard to tight junction protein expression as seen by immunofluorescence.

This study also demonstrates that the addition of the lignite extract supplement to the intestinal membranes can stabilize tight junction integrity in the face of PT-gliadin exposure. These results suggest a previously unrecognized biologic factor in the widespread development of tight junction dysfunction and the resulting disease epidemics in the developed world. The cumulative usage of antibiotics in humans and meat production coupled with the rapid use and accumulation of herbicides and pesticides in our agricultural system over the last 30 years has had a major impact on the bacterial biodiversity in the human gut [31]. These bacteria play a significant role in maintaining the tight junction integrity.

Because numerous products utilizing geologic sediments have tested positive for toxic levels of heavy metals, the lignite supplement was tested by ultra-sensitive mass spectrometry based heavy metal detection and found to be free of potentially toxic heavy metals or soil minerals [7,8]. It was further tested for toxicity on a cell type known to be very sensitive to toxins, namely human primary cultured RPTCs [15]. Results from this toxicity testing were surprising; in that even at very high levels of exposure, there was never any measurable toxicity. Even more unusual was finding improved vitality in the cultured cells as measured by a decrease in apoptosis.

Lignite extracts have been used in traditional medicine practices all around the world, including shilajit, humic acid, and fulvic acid. No studies have been found that examine these traditional lignite extracts on proximal tubule, small intestine, and large intestine cells in culture. Fulvic acid has been recognized to penetrate cell membranes and affect cellular biology more directly than the larger-particle humic and shilajit compounds. In this study we demonstrate that the lignite extract decreases apoptosis in the RPTC cultures, suggesting a unique safety profile.

The public health implications of these findings may be profound as gut membrane permeability via tight junction damage is increasingly being recognized as a root cause source for systemic inflammation and immune dysregulation [32]. The clinical manifestations of the 'leaky gut' phenomenon that are specific to the gliadin-mediated damage demonstrated in this study include the current epidemics of gluten sensitivity and celiac sprue. Tight junction injury is also implicated in a broad spectrum of seemingly disparate diseases including asthma, allergies, autism spectrum disorders, mood disorders, Parkinson's disease, and Alzheimer's dementia. There is on-going debate as to whether the apparent increases in the incidence of these conditions are simply reflective of increasing public and practitioner awareness and screening, or if in fact there has been a system wide environmental change in the food system and gut microbiome that have allowed for widespread vulnerability to tight junction damage. The data from this study demonstrate a clear mechanism by which the public's dietary consumption of refined wheat products may result in gut membrane permeability, unregulated antigen presentation to the GALT, and an increase in chronic inflammatory conditions in our population. Clinical studies are needed to establish the population response and potential clinical applications of lignite extract supplements in clinical practice.

Acknowledgement

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Competing Interests

DAR and ZB are shareholders and employees of Biomic Sciences, LLC, the entity that produces the lignite extract supplement used in the study. JJG is a consultant and shareholder of Biomic Sciences, LLC.

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